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CICLO XXXI

Analysis of novel tumor escape mechanisms from the Natural killer (NK) cells-mediated immune-surveillance

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1. NK cell functions

Natural killer cells (NK) are members of the Innate Lymphoid Cells (ILCs) family and represent approximately 5-15 % of circulating lymphocytes in healthy adults [1]. At first, they have been defined as large granular lymphocytes (LGL) for their size, larger than that of T and B lymphocytes, and *Natural Killers* for the presence, in their cytoplasm, of a cytolytic machinery composed by small granules containing perforin and granzymes, and for their ability to kill given targets in the absence of prior stimulation [2]. However, it is now evident that they require different stimuli to exert an optimal effector function [3], are involved in shaping adaptive immune responses and, although belonging to the innate immunity, they are capable of mounting a peculiar antigen-specific immunological memory, thus overcoming the original view of NK cells [4,5]. Along this line, a crucial regulatory role of NK cells has been demonstrated in both pathological and para-physiological events such as pregnancy (see 1.2 paragraph).

1.1 Cytolytic activity

Most studied function of NK cells is certainly represented by their cytotoxic activity against transformed cells such as tumor or virus-infected cells. However, they also contribute to the resolution of innate responses or to DC cells editing by exerting killing capability against autologous non-transformed cells such as neutrophils, macrophages and immature Dendritic Cells (DCs) [6,7].

The NK cytolytic activity is regulated by activating and inhibitory surface receptors whose integrated signals may overcome a "killing threshold" according to the rheostat model (Fig.1).

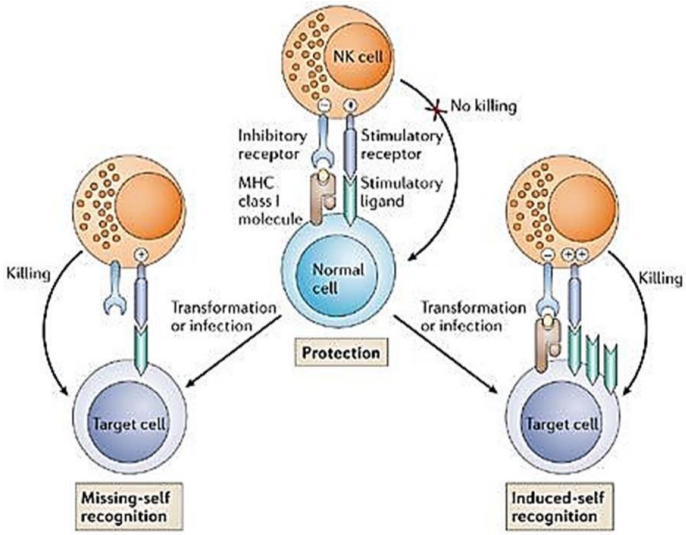

Figure 1. Protection or susceptibility to NK-mediated killing is the results of a balance between inhibitory and stimulatory signals in NK cells.

In a simplify view, normal autologous cells are spared since they express high levels of ligands of inhibitory receptors that control the activating signals [12]. Conversely, tumor transformation or infections lead to the downregulation/loss of inhibitory ligands and upregulation of ligands of the activating receptors ("danger signals") causing killing of target cells [8,9].

In human, the principal activating NK receptors are represented by NKp46 (CD335), NKp30 (CD337) and NKp44 (CD336) (collectively termed natural cytotoxicity receptors, NCR). Their expression is mainly restricted to NK cells and characterizes both $CD56^{dim}$ and $CD56^{bright.}$ NK cells, which represent the two most abundant circulating NK cell subsets in healthy donors. However, more recently studies have showed that other cell types express NCR, including innate lymphoid cells 1 (ILC1) (NKp46+) [10], plasmacytoid dendritic cells (NKp44+) [11] and a peculiar subtype of CD8+ T lymphocytes (NKp30+) [12].

NKp30 and NKp46 are expressed by not-stimulated (resting) and activated NK cells, whereas NKp44 represents an activation marker [13]. NCR are transmembrane proteins of type I with a short cytoplasmic tail lacking functional motifs capable of triggering intracellular signaling cascade. NKp46 is characterized by two Ig-like extracellular domains and a transmembrane portion with a positively charged residue (Arg), allowing its association with the ITAM (Immunoreceptor Tyrosine- based Activator Motif)-containing FcεRIγ and CD3ζ signal transduction molecules. These are also associated with NKp30, whereas NKp44 transduces activating signals thanks to the ITAM-bearing KARAP/DAP12 molecules. Both NKp30 and NKp44 are characterized by one Ig-like domain in their extracellular regions. In the last decade, several groups dedicated many efforts to discover the nature of the ligands expressed at the cell surface of transformed cells and specific for the activating receptors (Fig.2). NKp46 still remains an "orphan receptor", while NKp44 has been shown to recognize a novel isoform of the mixed-lineage leukemia (MLL5) protein[14]. NKp30 has been shown to bind B7-H6 molecule [15], although different experimental evidences suggest that NKp30 could recognize other, still unknown, tumor-associated ligand/s. Importantly, a functional cross talk occurs between the different NCRs, possibly resulting in the amplification of the activating signals [16].

Another receptor participating in the cytolytic activity of NK cells is NKG2D (CD314), which belongs to type C-lectin family and is also expressed by CD8+ TCR γ/δ e α/β T lymphocytes. In humans, NKG2D associates with DAP-10, an adaptive molecule containing in the cytoplasmic tail an YxxM sequence that, after phosphorylation, recruits the PI3K. NKG2D recognizes MICA/B and ULBPs, stress inducible molecules de-novo expressed after tumor transformation or virus infection[17].

Another crucial activating receptor is DNAM-1 (CD226), a transmembrane protein belonging to the immunoglobulin family [18]. PVR (CD155) and Nectin-2, two members of the Nectin family, have been identified as two ligands specific for DNAM-1 [19]. PVR plays a crucial role in determining the NK-related susceptibility of ex-vivo derived tumors and has also been shown to have a key role in cell motility during tumor cell invasion and migration [20]. More recently, PVR has been shown to be

recognized by TACTILE and TIGIT molecules, delivering activating and inhibitory signals, respectively.

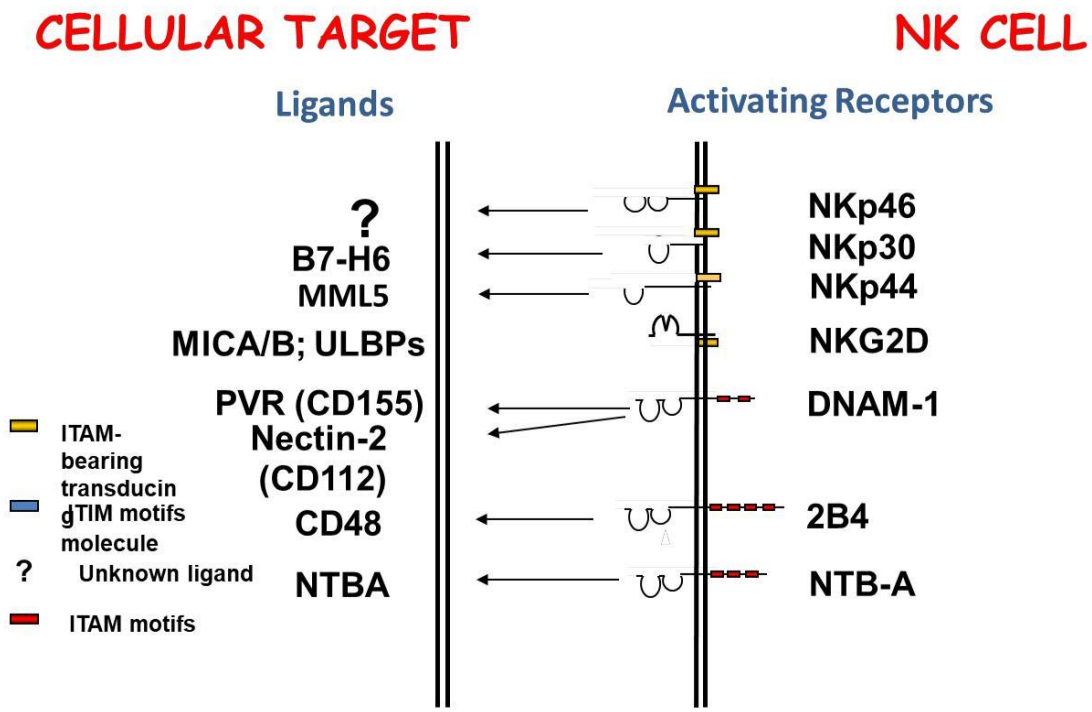

Figure 2. Principal non HLA-I specific activating receptors

Natural Killer cells express other triggering surface molecules that support the activity of the main activating receptors. These molecules, indicated as co-receptors, include 2B4 (CD244), NTB-A, NKp80 and CD59.

The activating function of NK cells is under the control of signals mediated by receptors that recognize self-HLA (Human Leukocyte Antigen) class I (HLA-I), molecules whose expression is high in normal healthy cells and decreases upon viral infections and tumor transformation [21]. These inhibitory NK receptors (iNKR) are represented by the inhibitory killer Ig-like receptors (CD158, iKIRs), which recognize HLA-A, HLA-B or HLA-C allotypes, the heterodimeric CD94/NKG2A complex, which recognizes non-classic HLA-I molecules (HLA-E), and LILRB1 with a broad HLA-I specificity. The iKIRs are clonally distributed and, together with CD94/NKG2A and LILRB1, create stochastic but tolerant repertoires of NK cells. In healthy individuals, the NK cells repertoire is selected during maturation in such a way that each NK cell expresses at least one iNKR for self-HLA class I molecules (self-iNKR). This phenomenon is termed classical “licensing” or “education” and provides the basis of self-tolerance. In developing NK cells, iNKRs are engaged, possibly in trans, by surrounding self-HLA-I expressing cells and transduce permissive signals that allow their phenotypic/functional maturation. Educated NK cells are self-tolerant but “armed”, becoming fully responsive to the engagement of activating receptors, exerting strong cytotoxicity against abnormal

targets and releasing large amounts of immunostimulatory cytokines. Lack of expression of self-iNKR results in the generation of "uneducated" NK cells that are detectable in small percentages in healthy individuals but functionally anergic.

Inhibitory KIRs (iKIRs) are type I transmembrane glycoproteins, with two or three extracellular Ig-like domains (KIR2D and KIR3D respectively) and a long intracytoplasmic tail containing Immune Tyrosine-based Inhibitory Motifs (ITIM). KIR2DL recognizes HLA-C and KIR3DL HLA-A/B allotypes, respectively (Fig.3). Regarding HLA-C allotype, the group 1 or group 2 alleles differ for the amino acid present in position 80 in the extracellular domain of the heavy chain and are recognized by different KIRs.

The engagement of iKIRs by their HLA-I ligands determines the phosphorylation of ITIM thus leading to the recruitment of Src homology 2 containing phosphatase 1 e 2 (SHP- 1 and SHP-2) [22], that inhibits NK cells activation.

CD94/NKG2A is an heterodimeric complex belonging to the C-type Lectin superfamily that, in the NKG2A intracellular portion, contains two ITIMs recruiting SHP-1 and SHP-2 after phosphorylation of tyrosine residues [23].

Activating counterparts of both iKIRs and CD94/NKG2A have been identified. The activating KIRs (aKIRs) (KIR2DS, KIR3DS) are highly homologous to the inhibitory ones in the extracellular portion whereas they present short cytoplasmic tails lacking ITIM and a charged transmembrane domain. They associate with the DAP12 signaling molecule that contains an Immunoreceptor Tyrosine-based Activation Motif (ITAM). The cross-linking of aKIR-DAP12 leads to NK cells activation through the recruitment of SYK and ZAP-70 protein tyrosine kinases.

The HLA-E specific heterodimeric CD94/NKG2C represents the activating counterpart of the CD94/NKG2A. Similar to aKIRs, CD94/NKG2C transduces activating signals through interaction with DAP-12.

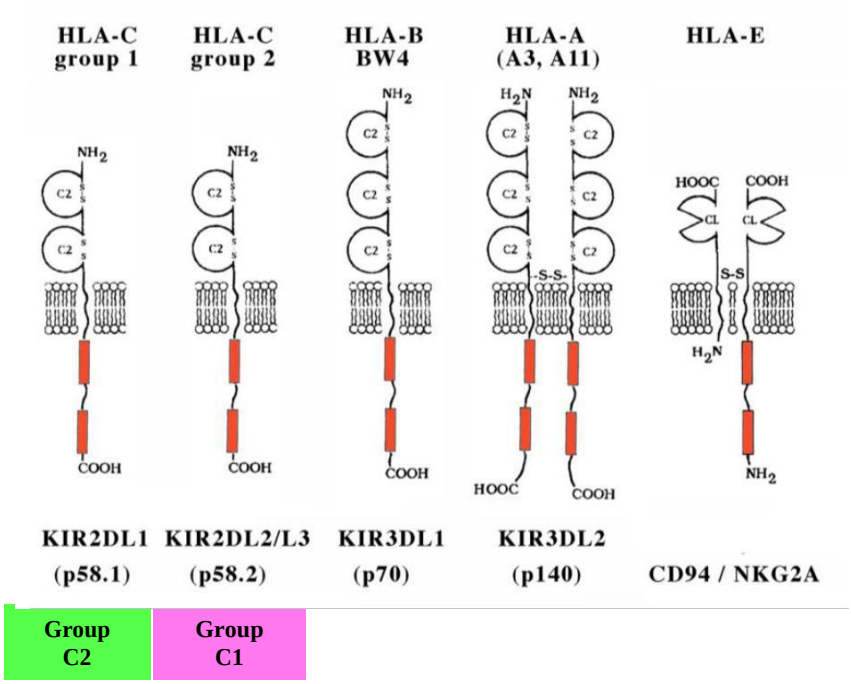

Figure 3. Principal MHC class-I specific inhibitory receptors

Natural killer cells are also capable of mediating Antibody Dependent Cellular-Cytotoxicity (ADCC) [24] (Fig.4). Antibodies (IgG), specifically reacting with surface antigens highly expressed by virus-infected or tumor cells, bind to CD16 (or FcγRIIIa), the low affinity Fc receptor expressed by Natural killer cells, unleashing cytotoxicity and target cells death. Importantly, ADCC seems to be more or less efficient depending on the CD16 isoform expressed by NK cells [25], an aspect that may deeply affect the outcome of antibody-mediated immunotherapies against tumors in certain individuals [26].

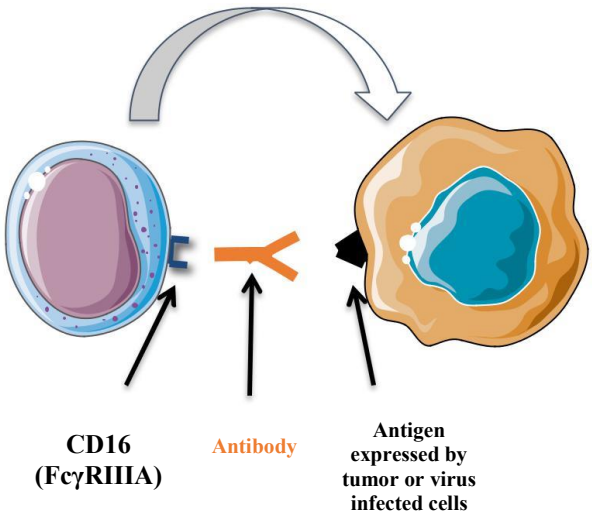

Figure 4. Antibody Dependent Cellular Cytotoxicity

CD16 is highly expressed by around 90% of circulating NK cells, whereas a minor fraction of circulating NK cells (and tissue-resident NK cells such as decidual NK cells) expresses very low levels or lack its expression. CD16$^{low/neg}$ NK cells are unable to mediate ADCC but, due to the constitutive expression of receptor for cytokines, produce large number of cytokines such as IFN-γ with immunoregulatory functions (see section 1.2) (Fig.5).

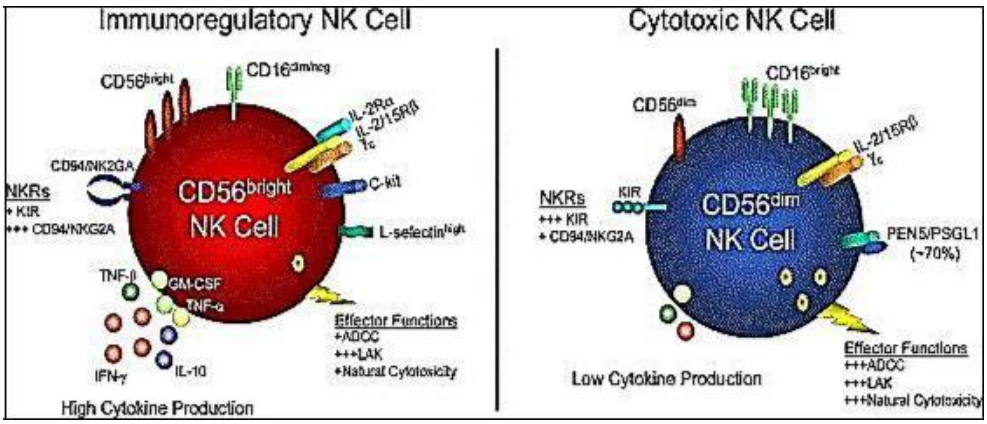

Figure 5. CD56^{bright} (red) and CD56^{dim} (blue) NK cells

CD16 $^{low/neg}$ and CD16^{high} NK cells express high or low levels of CD56 molecule (CD16^{bright} or CD16^{dim,} respectively) and differ for their migratory properties, since are equipped with a different repertoire of chemokine receptors [27].

CD16^{bright} CD16 $^{low/neg}$ express high levels of CC chemokine receptor 7 (CCR7) specific for CCL19/21 chemokines, involved in the migration to the secondary lymphoid organs, and L-selectin (CD62L), an adhesion molecule crucial for the interaction with high endothelial venules (HEVs) present in secondary and tertiary lymphoid organs. Moreover, they express high levels of CXCR3 and CCR5, that regulate the traffic of natural killer (NK) cells into peripheral tissues. On the contrary, CD56^{dim} CD16^{high} NK cells lack CCR7 and CD62L expression but express good levels of CXCR1 (receptor for CXCL1 or IL-8), S1P15 (receptor for S1P), CX3CR1 (receptor for CX3CL1 or fractalkine) and CHEMR23 (receptor for chemerin), essential for their migration to inflamed peripheral tissues or tumors (Fig.6.).

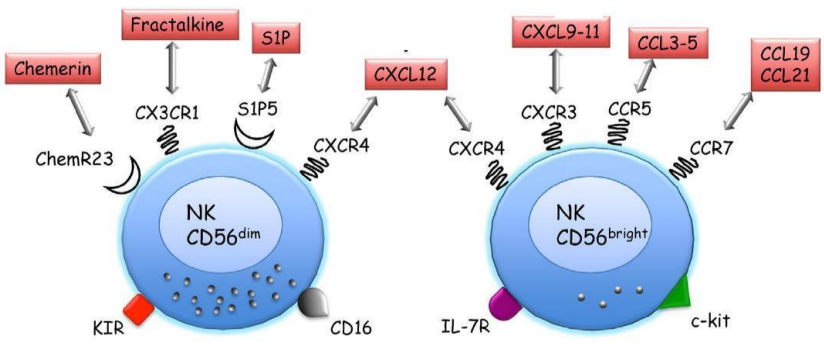

Figure 6. Chemokine receptor repertoire of CD56^{dim} and CD56^{bright} NK cells

1.2 NK cell Regulatory activity

Another important function of NK cells is represented by their ability to produce and release chemokines and cytokines, in particular IFN-γ, TNF-α and GM-CSF that regulate immune responses. An example of the importance of the regulatory activity of NK cells is their capacity to influence the activity of other effectors of innate immunity such as Dendritic Cells and Macrophages, which NK cells may meet in inflamed tissues [28]. After tissue damage or inflammation, as occurs upon virus infection or tumor invasion, NK cells are recruited in "perturbed" tissues by chemokines such as CXCL8, chemerin and CX3CL1, produced by different cell types present in the damaged sites [29]. The crosstalk between NK cells, macrophages and DCs, which release immunostimulatory cytokines such as IL-12, IL-15 and IL-18, induces NK cells activation with increased cytotoxicity and the release of IFN-γ and TNF-α [30,31]. These cytokines contribute to maturation of DCs, a process involving the engagement of NKp30 [32] , that recognizes a still undefined ligand. The DCs' maturation includes the upregulation of HLA-I, protecting DC from NK-mediated attack. DCs with defective maturation are efficiently killed by NK cells, which therefore contribute to the selection of the best APCs priming T lymphocytes toward Th1 polarized immune responses in secondary lymphoid tissues [33]. NK cells can also regulate the activity of mature DCs (mDCs) in secondary lymphoid tissues (SLT). Indeed, during DCs and macrophages crosstalk, NK cells can acquire CCR7 expression driving their migration to SLT. Despite this "late crosstalk" in humans needs further studied, in mice Fontecha et al demonstrated that, during immune responses, NK cells can be recruited in SLT via CXCR3, in a CCR7 independent way [34]. The recruitment of NK cells in lymph nodes was correlated with the induction of Th1 responses in vivo, since they act as an initial source of IFN-γ, fundamental for Th1 polarization. In vivo experiments with IFN-γ deficient NK cells unequivocally confirmed the relevant NK-mediated regulatory role in T cell polarization.

It is now established that IFN-γ and TNF-α, although optimizing Th1 responses, can also exert negative effects on anti-tumor activity by modifying tumor cells toward a phenotype that favors their escape from the immune surveillance, a process called "adaptive immune resistance" [35]. This can be based on the de-novo surface expression on cancer cells of protective ligands. These include different immune checkpoints molecules such as PD-L1 and PD-L2, recognized by the inhibitory PD-1 receptor expressed by NK (and T cells) (see for more details chapter 3,1 Factor dampening NK cell activity). Importantly, IFN-γ also upregulate PD-Ls on APCs [36]. In particular, IFN-γ released by NK cells induces in different hematopoietic cell lines and primary tumor cells (Multiple Myeloma, Acute Myeloid Leukemia and Acute Lymphoblastic Leukemia) a strong upregulation of PD-L1 (through the phosphorylation of JAK-1, JAK-2 and STAT-1) [37]which leads an increased resistance of tumor cells to the NK cell lysis. Killing can be restored by blocking the PD1-PD-L1 axis, using inhibitors blocking IFN-γ or of one or more transcription factors involved. Another relevant IFN-γ-mediated regulatory role for NK cells is represented by the upregulation of HLA-I expression on different kind

of tumor cells [38], which acquire resistance to NK cells killing, ac aspect that will be further discussed below.

A peculiar interesting regulatory role is that exerted by NK cells colonizing human decidua. Decidual NK cells (dNK) are low cytotoxic cells that show strong pro-angiogenic and tissue remodeling properties [39]. They are also involved in the induction of T regulatory lymphocytes (Treg), necessary to maintain the maternal tolerance towards the fetal allografts. dNK cells, representing the majority of lymphoid cells present in decidua in the first trimester of pregnancy, are characterized by a $CD56^{bright}$ $CD16^{neg}$ $KIRs^{pos}$ phenotype, but despite this their KIR repertoire is skewed toward recognition of HLA-C, the only classical MHC class I molecules expressed by trophoblastic cells [40]. dNK cells present a particular repertoire of other inhibitory receptors specific for HLA-E, G and C and express the inhibitory form of 2B4 receptor. These cells have low capability of forming immunological synapses and, despite the perforin and granzymes content, are poorly cytotoxic against classical targets. dNK cells, that could derive from CD34+ hematopoietic precursors present in human decidua, [41] produce angiogenic factors such as VEGF, release cytokines such as IL-8, SDF-1 and IP-10, and are involved in trophoblast growth, invasion and differentiation, as well as in the formation of new blood vessels . The proangiogenic properties of dNK have been confirmed *in vivo* in mouse models. After injection of human JEG-3 trophoblast choriocarcinoma cells and dNK cells, the mean size and weight of tumors were significantly greater, as compared to the tumors from mice inoculated with JEG-3 cells alone or with JEG-3 cells together with Peripheral Blood NK subsets [42]. Importantly, in pleural effusions of patients affected by inflammatory diseases or primary or metastatic tumors of different origin, NK cells can acquire a decidua like phenotype [43] , with the specific expression of surface markers like CD49a (an integrin alpha subunit that binds collagen and laminin) and CD9 (a tetraspanin protein family that plays a role in cell adhesion and cell motility), and a reduce expression of CD57, a marker of terminal differentiated and cytotoxic mature NK cells. Moreover, these NK cells derived from pleural effusions present less perforin content and a reduced degranulation capability in response to TGF-β1 and to both inflammatory and metastatic pleural effusions' fluids.

2. NK cells activity against cancer and cancer-associated tumor cells

Tumors are composed of a heterogeneous combination of cells, with different phenotypic characteristics and proliferative potential. The cytolytic activity of Natural killer cells is not limited to parenchymal tumor cells but they have the capability of recognizing and killing other cells present in the tumor microenvironment, In this context, chronic inflammation plays a critical role in cancer progression and some immune cell type types contribute in the growth and survival of tumor.

2.1 Cancer Stem Cells

In many type of tumors, two different subpopulation of cells have been described: CSC (cancer stem cells) characterized by stem cell properties, slow and asymmetrical growth, and more differentiated fast proliferating cancer cells [44]. CSC would be responsible of tumor generation/maintenance (they are also called tumor-initiating cells, TIC) and exert a crucial role in tumor recurrence, due to their resistance to irradiation and chemotherapy (Fig.7).

CSC represent an optimal therapeutic target to achieve complete eradication of the tumor, so many efforts focus on the discovery of strategies to identify and kill these cells. These include adoptive NK cells-based immunotherapeutic approaches. Indeed, it has been shown that NK cells efficiently kill tumor cells with stem cell like properties of tumors such as melanoma [45], glioblastoma (GBM) [46], breast [47] and colon carcinoma [48]. Preliminary reports considered NK cells inappropriate for adoptive immunotherapy, because GBM cells were shown to be resistant to lysis mediated by freshly isolated NK cells and NKL, a commonly used NK cell line [49,50]. These results were challenged by a study that, although confirming resistance of GBM cells to killing mediated by resting NK cells, showed that appropriate cytokine stimulation (IL-2 or IL-15) rendered NK cells highly cytolytic against both autologous or allogeneic GBM cells. Importantly, NK cells exerted a strong cytolytic activity against autologous GBM CSC cells expressing typical neural stem cells markers such as CD133 and c-KIT. Lysis was mainly due to the activity of DNAM-1 and NKp46 (although also NKG2D and NKp30 were involved) that prevailed on HLA-I-specific iNKR receptors. The high expression of PVR and Nectin-2 on the surface of GBM cells explained the main involvement of DNAM-1 in the killing. These data support the idea that NK cells could play an important role in the restraint of tumor recurrence and metastatization.

Regarding melanoma, a study revealed that primary CD133+ cells resulted more resistant to radiations than the CD133- counterpart, but both cell types expressed PVR, Nectin-2, ULBP-2 and MICA surface ligands specific for NK activating receptors, resulting equally susceptible to lysis mediated by IL-2 stimulated NK cells.

Interestingly, in colorectal carcinoma, CSC and non-CSC cells, derived from two cancer compartments, were differently susceptible to the action of fresh purified NK cells . CSC were more susceptible to lysis by NK cells than the relative differentiated cells, both in allogeneic and autologous

context. This peculiar behavior correlated with a higher surface expression, on CSC, of NKp30L and NKp44L and a lower expression of MHC-I, as compared to the differentiate tumor population. The same group has also demonstrated that NK cells were also able to recognize and kill breast CSCs, derived from human (and murine) breast cancer cell lines, *in vitro* and *in vivo*. In particular, CSCs enriched tumor spheres from the murine breast tumor cell line were more susceptible to the cytotoxic activity of NK cells than the parental cells. This correlated with a reduced surface frequency of inhibitory ligand H2-K and the high expression of PVR on the tumor spheres, confirming previous data on the crucial role of DNAM-1 in the recognition of CSCs. Tumor spheres were selectively killed even when freshly explanted murine NK cells were used as effectors.

Collectively, these results suggest that NK cells could be considered a potential weapon in therapeutic protocols aimed to limit tumor progression/recurrence by targeting the CSC compartment.

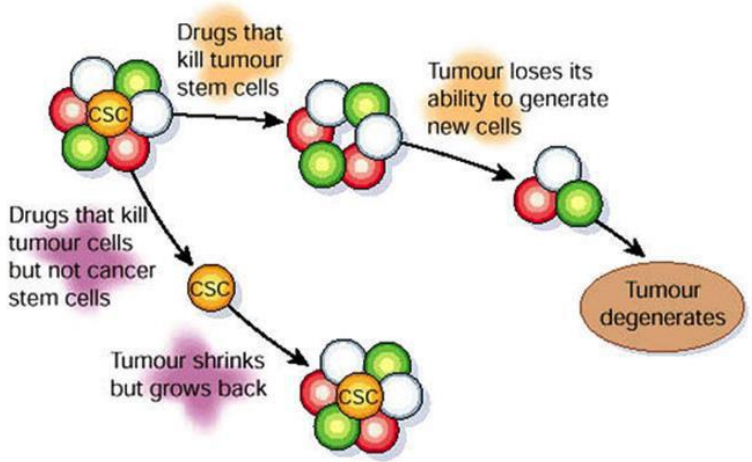

Figure 7. Cancer stem cells hypothesis from: "Stem cells, cancer, and cancer stem cells"

2.2 M2 macrophages

During inflammation NK cells can be interact with resident or recruited immune cells, including Dendritic Cells or Macrophages, influencing early innate and adaptive immune responses [51].

Monocytes can be recruited in tissues during inflammation and under the action of different soluble factors can differentiate in macrophages (M0 type)[52] and polarize toward M1 or M2, two different functional phenotypes. Macrophages polarizing towards M1 are characterized by an immunostimulatory behavior, release high amount of proinflammatory/immunostimulatory cytokines such as IL-1, IL-6, TNF-α, IL-12 and IL-23, and exert optimal antigen presenting activity. On the contrary, M2 show immunosuppressive and therefore pro-tumoral properties via different mechanisms. Although contributing to ECM deposition and tissues remodeling, M2 release/activate TGF-β (and IL-10) that inhibits the functions of NK cells and CD8+ lymphocytes, and produce factors promoting tumor vasculature and metastatization (VEGF or MMPs). Whereas M1 are protected thanks to the high expression of HLA-I, activated NK cells efficiently kill autologous M0 and M2

macrophages and NKp46 and DNAM-1 resulted to be the main activating receptors involved in killing .[53]

Importantly, in the context of pathogen-derived stimuli (e.s. LPS and BCG), a mutual fruitful crosstalk occurs between NK cells and M0 and M2 (but not M1-polarized macrophages). In particular, upon TLR stimulation macrophages polarize toward M1 and induce NK cells activation as demonstrated by the expression of CD25 and CD6, increasing the cytotoxicity against tumor cells and iDCs. Moreover, NK cells release IFN-γ, a process that requires the interaction of DNAM-1 and 2B4 with their ligands expressed on macrophage, as well as the action of macrophages-derived cytokines. A pivotal role is played by IL-18 that it is expressed as a membrane form by M0 and M2 and it is released as soluble form during M1-polarization. IL-18 is also responsible for the acquisition of CCR7 by NK cells [54]. On the other side, IFN-γ released by macrophage-activated NK cells supports polarization toward M1 and by increasing HLA-I expression protects M1 from NK-mediated attack. Importantly, a similar crosstalk occurs between NK cells and tumor associated macrophages (TAMs) isolated from ascites of patients with ovarian cancer [55]. Indeed, TAMs present a M2 like surface phenotype and show expression levels of PVR, Nectin 2 and HLA-I similar to in vitro IL-4 polarized M2 macrophages, derived from healthy donors. Accordingly, autologous IL-15 activated NK cells, derived from both ascites and peripheral blood and activated during NK/TAM crosstalk, were able to kill TAMs via DNAM-1 and NKp46.

2.3 Cancer-associated fibroblasts

Cancer associated fibroblasts (CAFs) are the dominant stromal components in the tumor microenvironment. These cells can have different origins: the majority of them derive from resident fibroblasts as a result of activation and genetic alterations [56], but their heterogeneity suggests that other cell types like adipocytes [57] , bone marrow derived stem cells [58], epithelial cells [59] (through epithelial mesenchymal transition) and endothelial cells (though End MT (endothelial-mesenchymal transition) could represent their source.

Their interaction with cancer cells is fundamental for the growth and the immune surveillance of tumor. In fact, CAFs are able to produce different growth factors and cytokines supporting the proliferation and the invasiveness of the primary tumor and the metastasis [60]. Among these, CAFs produce different pro-angiogenic factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2 and interleukin (IL)-8/CXCL8 [61]. Moreover, CAFs modify the ECM architecture through deposition of collagen and enhancement of cross-linking of collagen fibers, favoring the presence of fibrotic ECM, often correlated with a poor prognosis. Thanks to the Hyaluronan synthesis and the production of different Damage-associated molecular patterns (DAMPs), CSFs recruit macrophages into the stroma, where they are polarized toward M2. For

example, CAFs-mediated macrophages' recruitment into mammary carcinoma [62] and prostate cancer has been show to be dependent to secretion of CCL2 or CXCL14, respectively [63].

CAFs also modulate the activity of other immune cells present in tumor microenvironment, including T cells, thanks to the production of immune-suppressive cytokines such as TGF-β1, that impairs the acquisition of different T-cell effector functions, including anti-tumor cytotoxic [64].

TGF-β1 production is a hallmark of a subset of fibroblasts associated to non-small cell lung cancer (NSCLC)[65], which constitutively express PD-L1 and PD-L2, immune checkpoint ligands further upregulated by IFNγ. In three out of eight tumors analyzed, TGF-β1-producing CAFs (in the article indicate as TAF=tumor-associated fibroblast) co-cultured with autologous tumor associated T cells (TATs), suppressed their activation. However, in the other tumors analyzed, TAFs showed an opposite effect, stimulating TATs activation in a contact-dependent way, highly supporting the concept of phenotypic and functional heterogeneity of these cells.

Importantly, TGF-β1 has been described to represent a crucial autocrine mediator. Kojima and colleagues reported that resident human mammary fibroblasts, during the course of tumor progression, progressively shift toward a myofibroblast phenotype and acquire a TGF-β1 autocrine signal loop, necessary to maintain their tumor-promoting phenotype. This autocrine loop also involves stromal cell derived factor 1 (SDF-1, CXCL12) that mediates myofibroblasts differentiation. TGF-β1 and SDF-1 have also been found to be secreted by human prostatic CAFs [66]. In this tumor, TGF-β1 was able to increase the surface expression on tumor cells of CXCR4, the CXCL12 receptor, supporting the TGF-β1-mediated autocrine loop. Thanks to production of TGF-β1 and CCL5 (RANTES), CAFs have also been shown to contribute to the differentiation and recruitment of Treg cells into primary mammary tumors, stimulating cancer progression [67]. Moreover, several evidences suggest that CAFs, thanks to the production of TGF-β1, IL-6, IL-1β and CCL5, key cytokines for human Th17 differentiation, can also stimulate the generation and expansion of Th17 cells in different kind of tumors[68,69], thus promoting inflammation in the tumor microenvironment.

Shaping of the tumor microenvironment by the CAFs includes their influence on NK cells' phenotype and functions. It has been showed that, differently from normal fibroblasts, CAFs affect the cytolytic activity of NK cells against primary metastatic melanoma cells [70]. The phenomenon was paralleled by a significant dowregulation of NKp44, NKp30 and DNAM-1 expression, whereas the expression of NKG2D and NKp46 was only partially affected. The inhibition of DNAM-1 was contact-dependent, while Prostaglandin E2 (PGE-2), released by CAFs, was involved in the modulation of NKp30 and NKp44. Similar results were obtained in colorectal cancer [71]. In this experimental setting, NK cells co-cultured with purified CAFs presented a low surface expression of NKG2D, DNAM-1, NKp30 and NKp44, low expression of the CD69 activation marker and downregulation of cytolytic proteins such as perforins and granzymes. CAFs were also able to reduce the proliferation of NK cells and their capacity to release TNF-α and IFN-γ after activation. Also in this case, the high release of

PGE-2 by CAFs, as compared to normal fibroblasts, suggests its role in the modulation of main activities of NK cells.

2.4 Tumor-associated endothelial cells (TECs)

Tumor cells, in order to survive, proliferate and disseminate in the body, need adequate supply of oxygen and nutrients. Thus, an important hallmark of tumors is represented by neo angiogenesis [135]. Physiologically angiogenesis is a strongly controlled phenomenon that depends on chemical signals either stimulating or inhibiting angiogenesis. The prevalence of stimulating factors such as Vascular Endothelial Growth Factor (VEGF) occurs when specific sensors of oxygen relieve a hypoxic status inducing a regulated formation of new blood vessels (wounding or menstrual cycle). In tumor microenvironment, hypoxia stimulates an uncontrolled angiogenesis that supports the growth of tumor [72]. It has been demonstrated that tumor cells and TAMs can overproduce growth factors like VEGF, in order to have a constant supply of nutrients, necessary to maintain an accelerate metabolism [73]. Thus, angiogenesis is a critical phenomenon involved in the progression of the tumor, both primary and metastasis. This is the reason why, in recently years, different drugs have been developed targeting molecules and growth factors involved in the angiogenesis. Different soluble factors present in tumor microenvironment, like VEGF, FGF and TGF-β can promote angiogenesis, through the proliferation of tumor-associated endothelial cells and/or the recruitment from the BM of endothelial cells precursors (EPC). Tumor vessels present an altered anatomy as compared to the normal counterparts, an irregular shape and size as well as cytogenetic anomalies and different gene expression profile [74].

Recent studies have evidenced that tumor endothelial cells (TECs) can promote metastasis, producing proangiogenic factors [75]. In the initial steps of tumor metastasis, the production of biglycan, a small leucine-rich-repeat proteoglycan by TECs, facilities the trans endothelial migration of tumor cells, thanks to the interaction of tumor's byglican receptors TLR2 and TLR4. TECs, isolated from highly metastatic tumors, produced more byglican than those derived from low metastatic tumors, and the byglican levels in plasma was significantly lower in healthy donors than in cancer patients. Moreover, mice knockout for byglican expression and injected with highly metastatic TECs presented a low number of circulating tumor cells and lung metastases.

Different evidences suggest that TECs could modulate the activity of immune cells by an efficient cross talk with tumor cells [76,77]. Tumor cells derived from Lewis lung carcinoma produced soluble factors able to stimulate endothelial release of different immune suppressive factors [78]. In particular, endothelial cells pretreated with tumor-conditioned medium produce increased levels of PGE-2, VEGF and IL-6 and decreased levels of IL-12. In line with the hypothesis that VEGF is the main responsible of the modulation of endothelial cells functions, the block of VEGF through a selective inhibitor of the VEGF receptor tyrosine kinase prevented the increased secretion by lung endothelial

cells of PGE2 and IL-6. VEGF produced by endothelial cells was also involved in the downregulation of the production of IFN-γ by T cells. Again, blockade of the interaction between VEGF with its receptor restored the control levels of IFN-γ release.

To expand the scenario of immunomodulatory role of TECs, it has been showed in mouse models that recombinant VEGF, used at concentrations similar to those observed in advanced stage cancer patients, can shape T cell development [79]. In particular, VEGF can lead to a thymic atrophy and reduction of the number progenitors T cells in the thymus, contributing to the immune deficiencies observed in tumor. The same group also reported that a continuous infusion of VEGF in non-tumor bearing mice caused a decreased percentage of T cells in the spleen and lymph nodes [80].

The ability of VEGF to alter mature immune cells in a tumor context has been described for CD8+ T cells [81] and Dendritic Cells [82]. Analyses of human breast cancer showed inverse correlations between VEGF-A expression and $CD8^+$ T cell infiltration. Moreover, in many type of tumors, dendritic cells are defective in their capacity to present antigens. Indeed, the presence of high amount of tumor-derived VEGF in tumor microenvironment, which associates with a poor prognosis, contributes to inhibition of the DCs functions by inhibiting the DCs maturation and consequently their capacity to stimulate T cells.

A study carried out in mice showed that TECs can also modulate the activity of Natural Killer cells [83]. TECs express high levels of RAE, a mouse ligand of NKG2D. Engagement of NKG2D by the corresponding ligand causes its internalization leading to its disappearance from NK cell surface and to reduction of the NK cells–mediated antitumor activity. Moreover, normal endothelial cells treated with tumor-conditioned media were able to downregulate IFN-γ and TNF-α production by NK cells and to upregulate the release of the immunosuppressive cytokine IL-10. This strengthens the concept of the occurrence of a relevant crosstalk between tumor cells, TECs and NK cells [84] and confirm that TECs are important players in the mechanisms of immune regulation occurring in tumor microenvironment.

3. Factors Dampening NK Cells Activity

The capacity of NK to recognize and kill tumor cells and different tumor-associated cells that favor tumor progression (CAFs, M2-macrophages, and TECs) renders these immune cells promising effectors for immunotherapy. However, there is a growing evidence that NK-based therapeutic approaches must also consider that, in tumor microenvironment, different mechanisms could favor tumor escape from NK-mediated surveillance.

3.1 Membrane-Bound Molecules

The strongest NK-to-tumor contact inhibitory mechanism is represented by KIRs/HLA-I interactions (Fig.8). Indeed, it has been shown that in some instances the neoplastic cells (i.e. acute lymphoblastic leukemia (ALL) can retain high levels of HLA-I expression. Moreover, IFN-γ can increase the surface expression of HLA-I both further reducing their susceptibility to NK-mediated killing [85]. This scenario might easily occur in vivo and depend on effector cells number and/or cytokines' level. Indeed, it has been demonstrated that, when exposed to low numbers of NK cells, compatible with those infiltrating tumors, melanoma cells acquire resistance to NK-mediated aggression cells, mainly due to their high levels of HLA-I, resulting from IFN-γ activity [162]. Moreover, Propper et al showed that low doses of IFN-γ induced the expression of MHC-I in patients affected by metastatic melanoma [86]. Therefore, in certain tumors the inhibitory action of KIRs might prevail on the activating signals, leading to their protection from NK-mediated immune surveillance. In particular, therapeutic approaches in hematological and non-hematological malignancies, that include stem cell transplantation, select KIR/KIR ligand mismatched donors vs recipient combinations in order to ensure the onset in the recipient of NK cells donor endowed with "graft versus tumor" activity.

PD-1 is a receptor that has been described to be expressed and inhibited T cell activity [87]. It recognizes PD-L1 and PD-L2, expressed by Antigen Presenting Cells, and physiologically limits the immune response avoiding autoimmunity reactions. However, ligands can be expressed also by tumor cells. PD-1 has been detected also in NK cells and high percentages of PD-1+ NK cells have been described in cancer patients and in some healthy donors sero-positive for cytomegalovirus CMV [164]. In the last donors, representing about the 25% of all donors analyzed (200), PD1+ NK cells belong to $CD56^{dim}$, NKg2A ^{neg}, KIR^{pos}, $CD57^{pos}$ NK cells population. The functional analysis showed a reduce activity of PD-1+ NK cells, as compared to PD-1- cells, in terms of degranulation, capacity of performing ADCC and capacity to proliferate after exposure of rIL-2 and rIL-15. NK cell-mediated killing against PDLs+ tumors could be partially restored by PD1-specific mAbs and a complete recovering of killing was observed only by the combined masking of PD-1 and KIRs. The inhibitory PD-1/PDLs axis plays a pivotal role in recognition of tumors such as neuroblastoma that do not express detectable amounts of HLA-I. In this context, it has been shown that neuroblasts derived from bone marrow metastasis

significantly upregulate PD-L1 expression upon IFN-γ conditioning, with a more rapid kinetic as compared to HLA-I molecules. Importantly, PD-L1 has been shown to be constitutively expressed by different tumor histotypes and the evaluation of PD-L+ tumor cells in terms of percentage is a parameter predicting responsiveness to anti-PD1 therapies [88,89].

Another important inhibitory immune checkpoint is represented by the interaction between the B7-H3 ligand, an orphan ligand expressed by tumor, and its still unknown receptor(s). It has been shown that high expression of B7-H3 protects target cells from the NK-mediated cytotoxicity [90]. Indeed, the NK-mediated killing of metastasizing neuroblastoma (NB) highly expressing B7-H3 was deeply affected and significantly recovered by the use of a B7-H3 specific monoclonal antibody. The inhibitory role for B7-H3 has also be highlighted in the context of glioblastoma using a methodological approach based on B7-H3 specific siRNA [91] . Thus, B7-H3 has been recently included in the growing list of immune-checkpoints. B7-H3 is mostly tumor specific, is not significantly expressed by normal tissues and is highly expressed by tumor-associated endothelial cells. Importantly, B7-H3 has also been shown to promote tumor cells invasion and metastasis of different tumor hystotypes [92].

TIM-3, a member of the T cell immunoglobulin and mucin domain (Tim) family, represents an additional inhibitory receptor expressed by mature NK cells. It is also expressed by other immune cells, including CD4+ and CD8+ T. Once engaged by its ligand, galectin 9, which is expressed by different tumor cells, TIM-3 is able to reduce the functional activities of immune cells, including those of NK cells. A recent study in advanced melanoma [93] showed that circulating NK cells present a higher expression of TIM-3 and this is correlated with their exhausted phenotype. In patients with lung adenocarcinoma, blockade of TIM-3 recovered the capacity of NK cells to kill tumor cells and produce cytotoxic cytokines [94]. Homita et al. showed that about 75% of NK cells infiltrating GIST were positive for this receptor [95]. Importantly, all the GIST tissues with TIM-3 + NK cells were also positive for galectin 9. It is note that TIM-3 can also interact with the alarmin protein HMGB1 (High-Mobility Group Box 1), expressed by dendritic cells and different evidences suggest the presence of other still unknown ligand(s).

An additional inhibitory mechanism is represented by LAG-3 (lymphocyte activation gene 3), a transmembrane protein found on the surface on NK, T and B cells that belongs to immunoglobulin superfamily and recognizes with high affinity MHC class II molecules. Accumulating data suggest that, after activation, LAG-3 could negatively regulate different functions of immune cells, like activation and proliferation, and it could be involved in T cells exhaustion in cancer and chronic infections. Moreover, it seems to have a role in the immune suppressive activity of Treg. In NK cells, the data on the activity of LAG-3 are less clear. In LAG-3 deficient mice, NK cells presented reduced capacity to kill some tumor cell lines [96], suggesting that it could be crucial for NK function. On the

contrary, in human LAG-3 did not appear to be involved in NK cytotoxicity, because antibodies that blocked the molecule did not modify NK cells cytotoxicity [97].

TIGIT (T-cell immunoglobulin and ITIM domain) is an additional inhibitory receptor. It is expressed by NK cells, T cells (both effector and memory) and Treg cells. It recognizes CD155 (PVR) and CD112 (nectin 2), the same ligands of the activating receptor DNAM-1. It binds ligands with higher affinity, thus competing with DNAM-1 and negatively regulating the antitumor responses. In healthy donors, the expression of TIGIT on NK cells is heterogeneous, and TIGIT^{high} NK cells have reduced functional activities [98]. The expression of TIGIT on NK cells has been associated with NK cells exhaustion in tumor bearing mice [99]. Moreover, in patients affected by colon cancer, TIGIT was significantly higher in NK cells present in the intratumoral region as compared to NK cells in peritumoral site. TIGIT+ NK cells presented a lower IFN-γ and TNF-α production, as well as a lower expression of the lysosomal marker CD107a and of the cytotoxic molecule TRAIL, suggesting a reduced anti-tumor activity. Other studies confirmed that TIGIT is expressed by all human NK cells and that it can directly inhibit the cytotoxicity of NK cells thanks to its ITIM motifs [100]. Moreover, TIGIT can compete with DNAM-1 in the binding of PVR1 and Nectin-2 (CD112), but not Nectin- 3 (CD113).

All the above mentioned inhibitory axes are currently targeted by mAb-mediated therapies [101,102], Therapies are at different stages of clinical development, most of them are clinical trials in Phase I or Phase II, as occurs for Lirilumab, a monoclonal antibody specific for KIR2DL1/DL2 receptor. The most advanced immune interventions are represented by the use of FDA-approved antibodies targeting PD-1 or PD-L1 in different kind of solid and hematological malignancies. However, clinical responses are not observed in all treated patients and major efforts are now employed to discover biomarkers predicting responses to therapy. In this context, it has been shown a correlation between the microbiome-signature and clinical response to immune checkpoints blocking therapies, as discussed later.

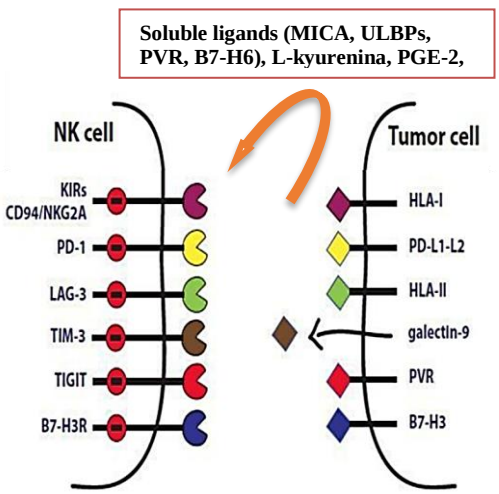

Figure 8. Principal membrane-bound and soluble factors limiting anti-tumor NK-cell activity

3.1 Soluble factors: TGF-β1, the prototypic immune suppressive factor

Tumors can evade the immune-surveillance mediated by NK cells thanks to the production of different soluble factors (Fig.8), that are able to modify, in NK cells, the expression levels of some receptors or to downregulate the main effector functions. These factors include soluble ligands of activating receptors (see section 1.1) that compete with the membrane-bound forms or induce downregulation of the counter receptors on NK cells surface.

Soluble MIC (sMIC) ligands are responsible of the downregulation of NKG2D in NK cells and CD8+ T cells infiltrating epithelial tumors [103]. Patients with tumors capable of releasing soluble MICA showed a strong downregulation of NKG2D on TILs, whereas NK cells of patients with sMIC negative tumors did not show this altered phenotype. The altered expression level of NKG2D was neutralized using a specific antibody binding sMIC or inhibitors of lysosomal pathway, confirming downregulation of NKG2D by endocytosis/degradation. sMIC, present in the serum of patients, had different sources deriving from tumor cell death, tumor-derived exosomes, proteases mediated shedding. A study analyzed the presence of NKG2D soluble ligands in serum of patients with melanoma metastasis. The authors found a strong correlation between the presence of sULBP2, but not sMICA, and disease progression. Both soluble ligands were more elevated as compared to healthy donors and high concentrations in the serum were associated with a reduced overall survival [104].

Soluble PVR (CD155), the main ligand of DNAM-1 activating receptor, has been detected in the serum of patients affected by a large variety of tumors (gastrointestinal, lung, breast and gynecologic) [105]. In patients, sPVR was present in higher concentrations as compared to healthy donors, and was significantly higher in those with advanced stages. In mouse model, the concentration of sCD155 was directly dependent of the weight of tumor, and, after the surgery, the concentration significantly decreased. Therefore, sCD155 may be considered a possible biomarker for cancer progression.

Another mechanism of immune escape, in particular from NK cells, is represented by soluble B7-H6. Elevated concentration of sB7-H6, the ligand of the activating receptor NKp30 [106], have been detected in blood serum of patients affected by melanoma, as compared to healthy donors. Importantly, the amounts of sB7-H6 in serum of patients correlated with the levels of expression of B7-H6 by patients' tumor cells. sB7-H6 has been detected in culture supernatants derived from different tumor cell lines (pancreatic carcinoma, liver carcinoma and melanoma). However, the downregulation of NKp30 was evident also after co-culture with tumor cells in which the presence of sB7-H6 was undetectable, suggesting that other factors, such as TGF-β1, might be responsible to the regulation of the expression of NKp30 (see below). ADAM-10 and ADAM-17 has been identified as the metalloproteases responsible for the sheading of B7-H6, and their inhibition increased the expression of B7-H6 on the surface of tumor cells, with increase activation of NK cells. It has been shown that the release of soluble B7-H6 by activated monocytes and neutrophils can be induced, in inflammatory conditions, in particular by the action of proinflammatory cytokines, like IL-1 and IL-6.

Galectin 9 has been identified as the main ligand of TIM-3, being TIM-3/galectin 9 an immune checkpoint axis involved in the downregulation of the response of T cells and NK cells. Galectin 9 is a member of a family of carbohydrate-binding protein and can be released in soluble form by a large array of tumors [107]. In humans and mice, galectin-9 upon TIM-3 engagement can impair the main NK cells functions, including cytotoxicity and cytokines production [108].

Recent studies showed that an important role in the regulation of the immune activity of NK cells is played by catabolic molecules derived from the amino acid Tryptophan (Trp),[109], in particular by the enzyme indoleamine 2,3-dioxygenase (IDO). IDO is produced by different kind of tumors, and upregulation of its activity has been correlated with tumor progression [110]. Among the catabolites generated by IDO and endowed with immunomodulatory activity against Natural Killer Cells, it should be mentioned L-kynurenine, which has been demonstrated to prevent the IL-2-induced upregulation of NKp46 and NKG2D activating receptors in NK cells, both at protein and mRNA levels [111]. This negative regulation significantly affected the activating receptor-mediated capability of killing of tumor cells and of releasing cytokines. IDO-derived catabolites are effective molecules also used by Dendritic Cells, Mesenchymal stem cells (MSC), and tumor associated fibroblasts to shape NK cells proliferation and functions [112] . Other soluble factors showing an inhibitory activity against NK cells are prostaglandin E2 (PGE2), macrophage migration-inhibitory factor (MIF) and adenosine. The first is a small lipid molecule, produced by different kind of tumors cells and engaging the Prostaglandin E2 receptor 4 (EP4), whose targeting in mouse model was shown to prevent the inhibition of the main activities of NK cells leading to a better control of breast cancer. MIF, a factor involved normally in the survival of macrophages is constitutively produced by different tumor histotype, like ovarian cancer [113], melanoma [114] and uveal melanoma, and exert an inhibitory effect on NK cells that could be efficiently contrasted by a specific monoclonal antibody. In ovarian cancer, one of the MIF-mediated mechanisms leading to NK suppression is represented by the downregulation of NKG2D expression at transcriptional level. Adenosine, another soluble molecule present in tumor microenvironment, is an endogenous purine nucleoside that mediates various biological effects by binding to adenosine receptors (AdoRs), present in 4 different isoforms (A1, A2a, A2b, A3),. Concentrations of adenosine similar to those produced in tumor microenvironment by Sarcoma cells and Lewis lung carcinoma cells in hypoxic conditions, inhibited main functions of murine NK cells [115]. These effects have been confirmed in human, in which it has been demonstrated to be due to the adenosine-mediated engagement of Adenosine Receptor 2A (AdoR2A) [116].

Among the plethora of soluble factors negatively regulating the NK cells mediated activity, Transforming Growth Factor beta (TGF-β) plays a strong and pleiotropic pivotal role.

TGF-β1, the most studied isoform, is released by a large array of cell types including immune cells. It is a physiologic regulator controlling the activation and the proliferation of different cell types, including macrophages and T lymphocytes, regulating the amplitude of immune responses and

maintaining peripheral tolerance [117]. According to its functions, TGF-β1 knocked-out mice showed multiorgan inflammation and early death [118]. TGF-β1 also contributes to the development of regulatory FoxP3+ lymphocytes and Th17 lymphocytes in the context of infections by pathogens [119]. Importantly, the in vivo TGF-β1 activity is finely regulated. TGF-β1 has been called a "sleeping giant" [120], since it is available in large amounts but inactive in the body. Indeed, after intracellular synthesis and processing is released in a Small Latent Complex (SLC), non- covalently bound to the protein LAP, or in a larger complex called Large Latent Complex (LLC), formed by SLC covalently bound to LTBP protein called Latent TGF-β-Binding Protein. Notably, a specific region of LTBP shows binding sequences for extracellular matrix (ECM) with important consequences on TGF-β1 localization within ECM. Different stimuli (heat, acidic PH, Reactive Oxygen species, various proteases, shear stress or binding with some integrins) allow the release of TGF-β1 from complexes. The free active form is capable of interacting with its receptors TGF-RI and RII, leading to the phosphorylation of intracellular SMAD molecules, regulating NK cells development and functions [121]. NK cells derived from peripheral blood of healthy donors and cultivated in the presence of TGF-β1 drastically reduced their cytolytic activity against tumor cell lines, in a concentration dependent manner[122]. Moreover, TGF-β1 is capable of modulating in NK cells the constitutive and the IL-2 driven expression of the activating receptors NKG2D and NKp30 [123]. A reduction of the corresponding transcript was detected for NKp30 but not for NKG2D. Accordingly, TGF-β1 has been shown to downregulate DAP10 protein, a signal transducing molecule essential for NKG2D expression in human [124]. The reduced levels of NKG2D and NKp30 resulting from TGF-β1 conditioning significantly affected the NK-mediated killing of different tumor cell lines and/or of autologous immature myeloid DCs, likely impacting on the selection of iDC undergoing successful maturation. Importantly, NKG2D downregulation has been documented by in NK (and CD8) cells isolated from patients affected by glioblastoma multiform [125]. NKG2D expression was recovered after tumor resection, an observation that correlated with an increased killing of NKG2D-positive tumor targets. In this study, blockade of TGF-β1, differently from blocking of soluble ligands for NKG2D, was sufficient to restore the NKG2D levels. Accordingly, high levels of TGF-β1 were detected in the patients' serum that could be produced not only by tumor cells but also by regulatory T cells, present in high percentage in these patients. An in a vitro model showed that a chronic interaction between IL-15 stimulated human NK cells and colorectal tumor cell lines led to an exhaustion of NK cells characterized by a strong reduction of NKp30, NKG2D and DNAM-1 and a reduced production of IFN-γ. This chronic inhibition was mediated by TGF-β1, and the use of TGF-β1 antibody restored the main NK functions. NK cells with exhausted phenotype were detected in ascites of patients affected by ovarian cancer but not in their peripheral blood [126].

The same exhausted phenotype could be induced in blood-NK cells after coculture with autologous tumor cells, and the correct levels of activating receptors could be restored using a specific TGF-β1

antibody, confirming its important role in affecting NK cells functions in tumor microenvironment in vivo. Along this line, TGF-β1, in concentrations similar to that observed in tumor microenvironment of AML patients, was able to progressively impaired the killing capability against myeloid leukemia cells of ex vivo expanded NK cells, which showed a progressive decline in the expression of NKG2D and CD16 [127].

TGF-β1 can also be expressed as a membrane form as occurs in Myeloid derived suppressor cells (MDSC), a myeloid population strongly expanded during tumor progression and able to inhibit different immune cells including T cells, DCs and NK cells. The inhibition mediated by the Membrane-bound form of TGF-β1 is the main responsible for the suppression of the NK cells functions in liver cancer [128].

It is well known that TGF-β1, activating STAT transducing molecules, also reduces the production of IFN-γ and TNF-α induced by the synergistic effect of different proinflammatory cytokines such as IL-12, IL-15 and IL-18. Moreover, recently a new regulatory function for TGF-β1 has been demonstrated. Both rTGF-β1 and TGF-β1 released by neuroblastoma cell lines were capable of modulating the chemokine receptor repertoire of NK cells [129]. In particular, it upregulated the expression of CXCR4 and CXCR3, while downregulating that of CX3CR1. On the contrary, the expression of CCR7 and CXCR1 was unaffected. The decreased expression of CX3CR1, that plays a role in NK cell adhesion to endothelium and extravasation, may hamper their recruitment to peripheral tissues, including tumor sites.

AIMS OF THE STUDY

During my PhD program at University of Genova my research activity has been focused on the analysis of the anti-tumor activity of human NK cells and of the mechanisms that may limit or enhance their function.

The topics have been summarized and critically discussed in reviews (*publication n°1, 2, 3*) and new data published in research articles. In particular, I have analyzed the activity of cytokine-stimulated NK cells against tumor-associated endothelial cells, isolated from bone marrow aspirates of MM patients with active disease (MMECs) (*publication n°4*); investigated the immunological off-target effects of imatinib and nilotinib, tyrosine kinase inhibitors (TKIs) used in the clinical management of hematological neoplasms that have also showed benefits in solid tumors such as stage 4 neuroblastoma (NB) *(publication n°5);* analyzed the miRNA involved in the TGF-β1-mediated modulation in NK cells of CX3CR1, a chemokine receptor that drives these effectors toward peripheral tissues, including tumor sites. *(publication n°6)*

More recently, my efforts have been dedicated to the following aims:

AIM 1. Identification of the mechanisms strengthening or contrasting TGF-β1-mediated modulation of NK cells activity.

AIM 2. Analysis of the impact of gut microbiome on immunotherapy. Experiments have been carried out during my stay at the Institute Gustave Roussy (Villejuif, France) (January 2018-October 2018) under the supervision of Prof. Laurence Zitvogel.

These studies are described below and manuscripts are in preparation.

AIM 1

Results

IL-18 boosts the immunomodulatory effects of TGF-β1 in NK cells

As previously shown [123,129], optimal concentrations of TGF-β1 induced down-regulation of the NKp30 and NKG2D activating receptors and modified the chemokine receptor repertoire of peripheral blood (PB) NK cells. In particular, it significantly increased the expression of CXCR4 and CXCR3 and decreased that of CX_3CR1 (Fig.1). In search for cytokine(s) that could modify the TGF-β1-mediated immunomodulatory effects on human NK cells, we cultured PB NK cells in the presence of TGF-β1 alone or in combination with different cytokines known to exert immunostimulatory functions (Fig.1).

IL-2 and IL-15 significantly contrasted the TGF-β1-mediated down regulation of the activating receptors, which recovered expression levels comparable (or even higher) to untreated NK cells. On the contrary, IL-21, IL-12, IL-27 and IL-32 had no effects. IL-18 was unable to restore NKG2D expression (Fig.1), despite its ability to upregulate its expression when used alone (Fig.2). Notably, IL-18 showed an unexpected immunomodulatory role, significantly strengthening the TGF-β1-mediated downregulation of NKp30 surface expression (Fig.1).

Regarding the chemokine receptor repertoire, none of the cytokines analyzed influenced the effect of TGF-β1 on CXCR3 expression. IL-2 and IL-15 did not alter the effect of TGF-β1 on CX_3CR1 while strongly contrasted the CXCR4 up-regulation (Fig.1), leading to chemokine receptor's surface levels even lower than those present in untreated NK cells. Again, IL-18 showed a very peculiar behavior. Indeed, it significantly potentiated the activity of TGF-β1 further reducing the expression of CX_3CR1 and increasing that of CXCR4 (Fig.1). The latter effect was evident in both $CD56^{dim}$ and $CD56^{bright}$ NK cells. Notably, a significant decrease of CX3CR1 surface levels was detected also when IL-18 was used alone (Fig.2). Morever, IL-18 also decreased the expression of CXCR1, both when used alone or in the presence of TGF-β1 (Fig.1 and Fig.2).

Among the other cytokines analyzed, IL-21 significantly counteracted the TGF-β1-mediated up-regulation of CXCR4, although to a lower extent as compared to IL-2 and IL-15 (Fig.1). On the other hand, IL-21 shared with IL-18 the property of further reducing the CX_3CR1 surface levels resulting from TGF-β1 conditioning. IL-12, IL-27 and IL-32 did not modify the TGF-β1-mediated modulatory effect on the chemokine receptor repertoire (Fig. 1 and Fig.2).

It is of note that IL-18 significantly potentiated the activity of TGF-β1 on NKp30 and CXCR4 expression but had no effect on these molecules when used alone (Fig.1). Moreover, IL-18 alone shared with IL-15 classical immunostimulatory functions such as upregulation of NKG2D, CD69 and CD25 surface levels (Fig. 2). The additive effect of IL-18 on TGF-β1-mediated up-regulation of CXCR4 was preserved using TGF-β1 concentrations ≥1 ng/ml (Fig.3).

On the contrary, that on NKp30 surface levels was lost using suboptimal concentrations of TGF-β1 (Fig.3).

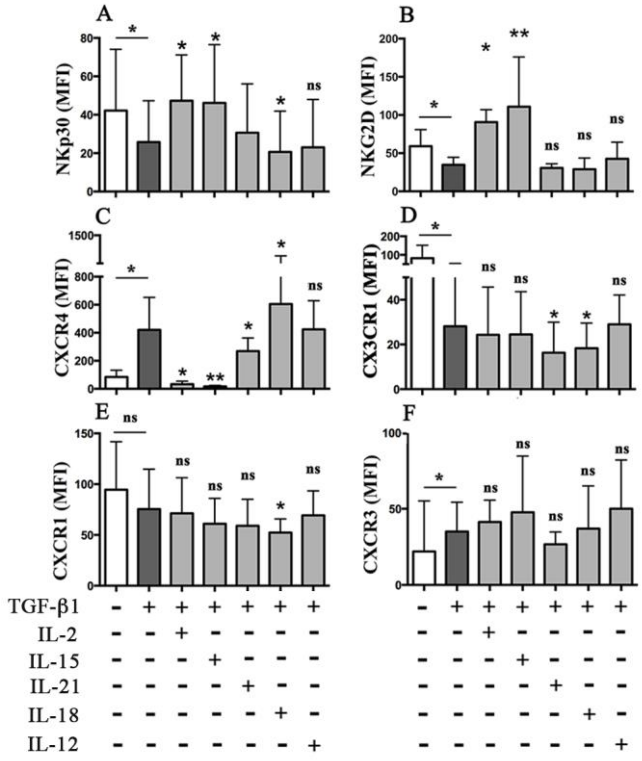

Figure 1. Effect of pro-inflammatory cytokines on the main TGF-β1-mediated immunomodulatory effects

NK cells purified from peripheral blood of healthy donors (PB NK) were cultured for 48 hours either in the absence (control, CTR, white bars) or in the presence of TGF-β1 used alone or in combination with the indicated cytokines. Cells were analyzed by flow cytometry for the expression of activating and chemokine receptors. Average of 6 independent experiments (6 unrelated healthy donors). Mean fluorescence intensity (MFI) and 95% confidence intervals are shown. **p<0.01 *p<0.05; ns means p not significant. If not indicated, statistical significance is referred to results obtained with TGF-β1 alone.

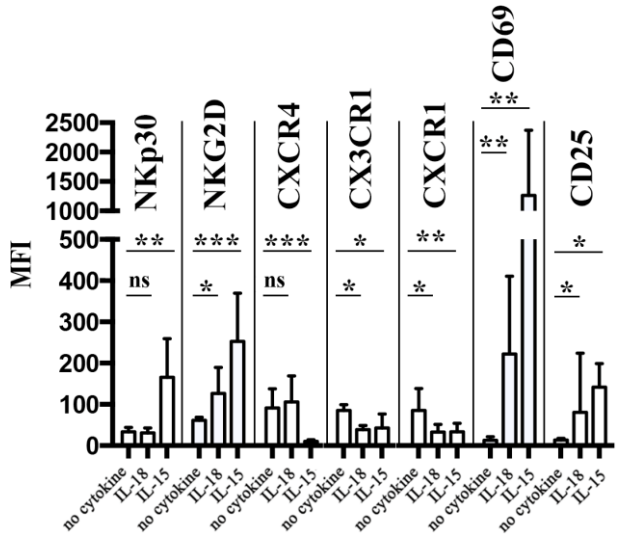

Figure 2. Effect of IL-18 or IL-15 on the expression of activating and chemokine receptors

PB NK cells, cultured for 48 hours either in the absence (CTR) or in the presence of IL18 or IL-15, were analyzed by flow cytometry for the expression of the indicated molecules. Average of 6 independent experiments (6 donors). Mean of MFI and 95% confidence intervals are shown. *p<0.05, **p<0.01; ***p<0.001; ns means p not significant.

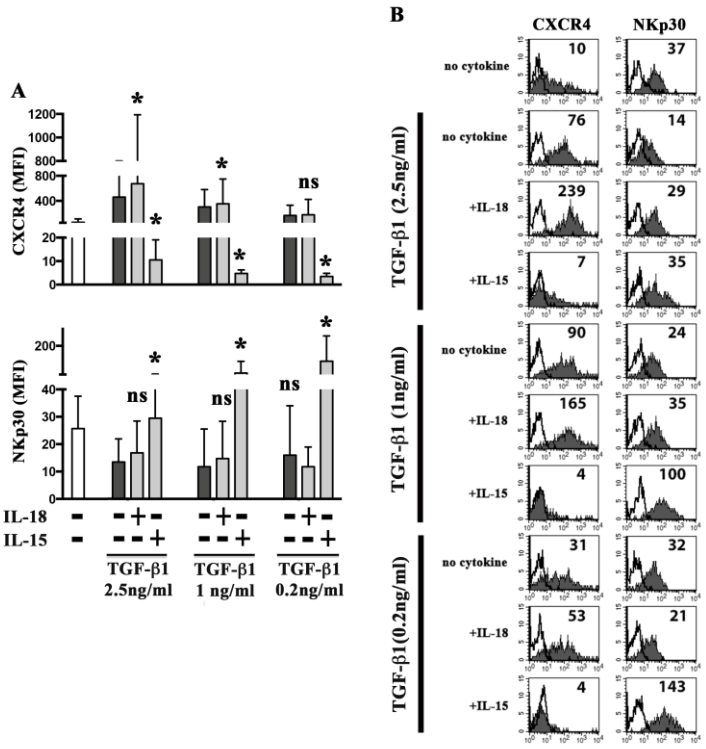

Figure 3. Analysis of the effects of IL-18 in combination with sub-optimal doses of TGF-β1

A) Cytofluorimetric analysis of the expression of CXCR4 and NKp30 in PB NK cells cultured for 48 hours in the absence (CTR, white bars) or in the presence of suboptimal doses of TGF-β1 alone or in combination with IL-18 or IL-15. Average of 5 independent experiments (5 donors). Mean of MFI and 95% confidence intervals are shown. *p<0.05; ns means p not significant. Statistical significance is referred to results obtained with TGF-β1 alone.
B) Representative experiment of the cytofluorimetric analyses shown in panel A. White profiles refer to cells incubated with isotype-matched mAbs. Values inside each histogram indicate the MFI.

TGF-β1 plus IL-18 affect NKp30-mediated triggering

We analyzed whether the very low NKp30 surface densities resulting from TGF-β1 plus IL-18 conditioning impacted on the killing capability of NK cells. To this end, PB NK cells were treated with the cytokines combination and analyzed in ADCC against the FcγR+ P815 target cells. As shown in fig. 4A, differently from untreated cells, the mAb-mediated engagement of NKp30 in TGF-β1 plus IL-18 conditioned NK cells was virtually unable to induce target's lysis. On the contrary, the engagement of CD16 still induced optimal cytotoxicity. This result suggested that the combined action of these cytokines selectively affected the triggering capability of NKp30 rather than significantly impacting on the overall cytolytic potential of NK cells (i.e. granzyme/perforin cellular content).

Next, we analyzed whether classical immunostimulatory cytokines could counteract the TGF-β1 plus IL-18 immunomodulatory effects. To this end, PB-NK cells were treated with TGF-β1 plus IL-18 in combination with either IL-2 or IL-15 (Fig.4). Both cytokines induced in NK cells the recovery of the NKp30 surface expression (Fig.4B). This was paralleled by restoration of

receptor's function. Indeed, mAb-mediated engagement of NKp30 resulted in a strong cytolytic activity against target cells, significantly higher as compared to untreated PB-NK cells (fig.4A). Finally, IL-2 and IL-15 abrogated the CXCR4 up-regulation induced by TGF-β1 plus IL18 (Fig.4B).

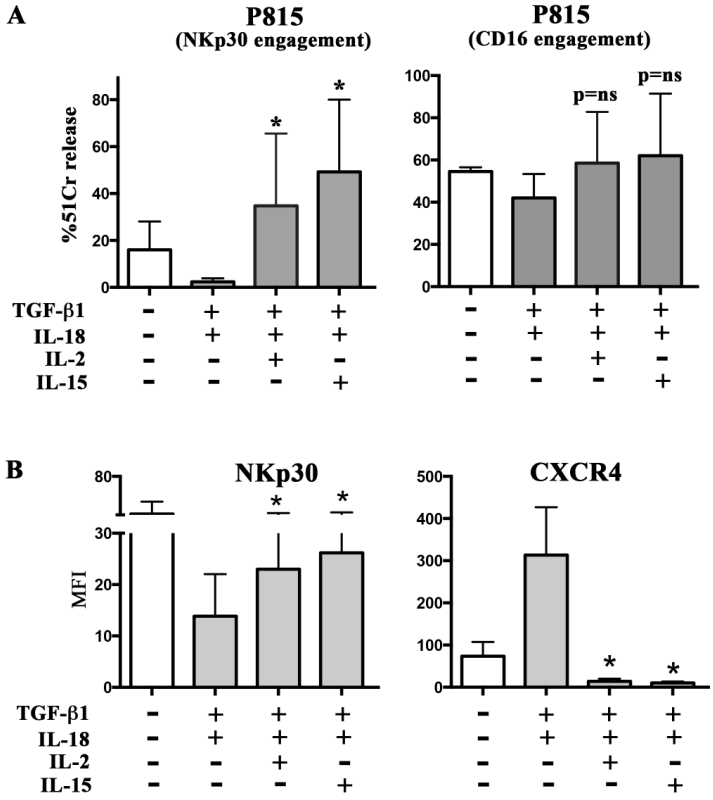

Figure 4. IL-2 or IL-15 strongly contrast TGF-β1 plus IL-18-mediated modulatory effects

A) PB NK in the indicated culture conditions were analyzed for cytolytic activity (^{51}Cr release) against the FcγR+ P815 target cell in the presence of mAb specific for NKp30 or CD16.
B) Cytofluorimetric analysis of NKp30 and CXCR4 expression in PB NK cells treated with the indicated cytokines combinations. Average of 4 independent experiments (4 donors). Mean of MFI and 95% confidence intervals are shown. *p<0.05; ns means p not significant

Molecular mechanisms involved in the IL-18-mediated additive effect on TGF-β1 activity

To analyze whether transcriptional regulation was involved in the IL-18-mediated additive effect on TGF-β1 activity, PB NK cells were treated with different concentrations of TGF-β1 in the absence or in the presence of IL-18 or IL-15 and analyzed by quantitative PCR for CXCR4 and NKp30 mRNA expression. qPCR for CD25 mRNA was also performed as control. As shown in Figure 5, treatment with optimal doses of TGF-β1 resulted in an increase of CXCR4 mRNA and reduction of NKp30 mRNA, effects that were significantly potentiated by IL-18. The addition of IL-18 to suboptimal doses of TGF-β1 (2,5 ng/ml) did not cause any significant change in NKp30 mRNA levels, according to results on receptor expression depicted in Fig.3. On the contrary,

although significant up-regulation of CXCR4 at protein level was detect upon addition of IL-18 to suboptimal doses of TGF-β1 (both at 2,5 and 1 ng/ml) (Fig.3), this effect did not seem to correlate with increased CXCR4 mRNA levels, suggesting the release to the cell surface of preformed cytoplasmic CXCR4 (Fig.5)

As expected, IL-18 alone caused a significant up-regulation of CD25 mRNA, an effect shared by IL-15. Moreover, IL-15 contrasted the effect of TGF-β1, significantly increasing NKp30 and reducing CXCR4 mRNA levels (Fig.5).

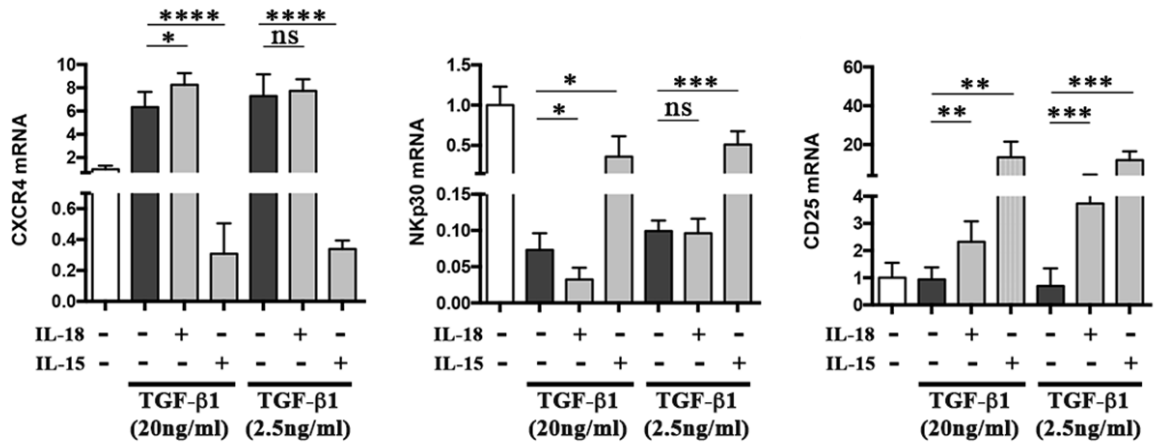

Figure 5. CXCR4, NKp30 and CD25 mRNA expression in NK cells treated with TGF-β1 plus IL-18 or IL-15

PB NK cells were treated for 48h with the indicated amounts of TGF-β1, in the absence or in the presence of IL-18 or IL-15, and analyzed by qPCR for CXCR4, NKp30 and CD25 mRNA expression. Average of 5 independent experiments (5 donors, each analyzed in triplicate). GAPDH has been used as reference control.

p38 MAPK and MEK/ERKs are kinases involved in the transduction pathway of IL-18 and TGF-β1 . To analyze their possible involvement in the additive effect of IL-18 on TGF-β1 activity, PB NK cells were conditioned with these cytokines, in the absence or in the presence of specific kinase inhibitors (Fig.9), and analyzed by flow cytometry for NKp30 and CXCR4 expression (Fig.6 and Fig.7). The capability of TGF-β1 alone to modulate NKp30 and CXCR4 expression was not significantly affected either by p38 MAPK or MEK/ERKs inhibitors, suggesting a predominant intervention of the SMAD signaling pathway. Differently, p38 MAPK and MEK/ERKs were involved in the additive effect of IL-18 on TGF-β1. In particular, the p38 MAPK inhibitor abolished (NKp30) or significantly reduced (CXCR4) the additive effect of IL-18 (Fig.6 and Fig.7). That on NKp30 down-regulation was also significantly affected by inhibition of MEK/ERKs . On the contrary, MEK/ERKs inhibition did not cause significant variation in up-regulation of CXCR4 (Fig.6 and Fig.7).

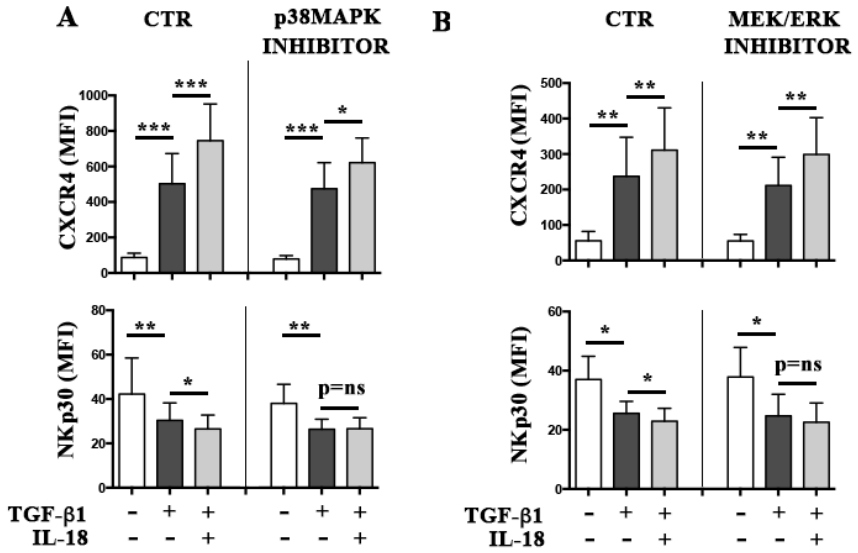

Figure 6. Effect of p38MAPK or MEK/ERK inhibitors on the additive effects mediated by IL-18 on TGF-β1 activity

Cytofluorimetric analysis of CXCR4 and NKp30 surface expression in PB NK cells treated with TGF-β1 or TGF-β1 + IL-18, in the absence (CTR) or presence of p38MAPK inhibitor (panel A) or MEK/ERK inhibitor (panel B). Average of 4 independent experiments (4 donors). Mean of MFI and 95% confidence intervals are shown. *p<0.05, **p<0.01, ***p<0.001; ns means p not significant.

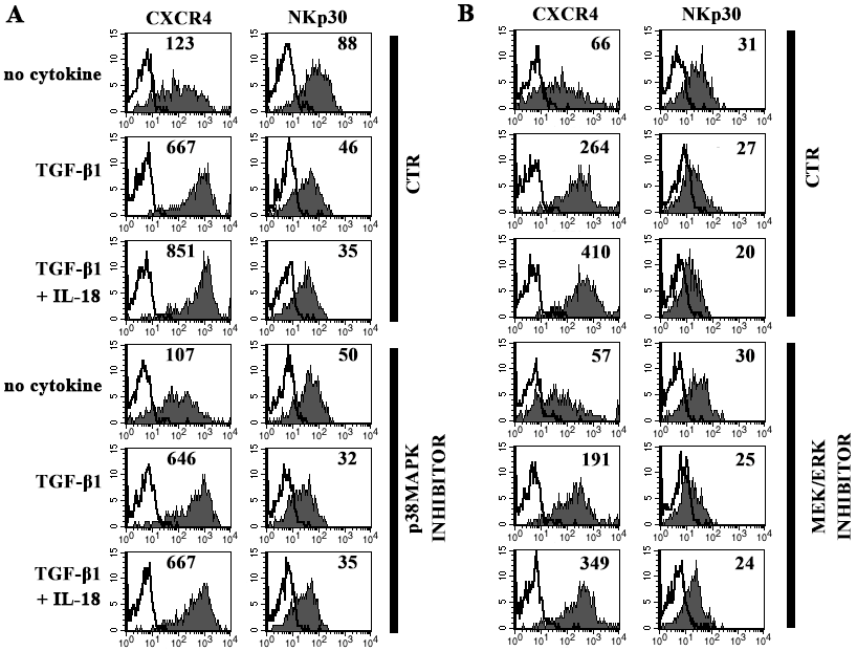

Figure 7. Effect of p38MAPK or MEK/ERK inhibitors on the additive effects mediated by IL-18 on TGF-β1 activity

A, B Representative experiment of the cytofluorimetric analyses shown in Fig.9. White profiles refer to cells incubated with isotype-matched mAbs. Values inside each histogram indicate the MFI

Effect of IL-18 plus TGF-β1 on NK cell proteome

To gain insight on the effects of TGF-β1 and IL-18 on NK cell phenotype and function we used a proteomic approach. PB-NK cells untreated (CTR) or conditioned for 48 hours with TGF-β1, alone or in combination with IL-18, were lysed and analyzed by high-resolution mass spectrometry. As control, NK cells were analyzed for receptors expression by flow cytometry (data not shown). Data processing through the MaxQuant software allowed the identification of a total of 4331 proteins (of which 3971 were quantified using a Label-Free Quantitation approach).

In order to analyze the statistically significant regulated proteins, two volcano plots were generated from TGF-β1 vs CTR and TGF-β1 plus IL-18 vs TGF-β1 data sets, and representative proteins selected on the basis of a two-sample t-test (FDR = 0.05 and s0 = 0.1 log10 of P value, y-axis) (Fig.8). By this selection, several proteins have been identified, involved in different cellular processes including innate and adaptive immune responses, cell-to-cell adhesion, responses to external stimuli, exocytosis (SANKEY) (Fig. 9). Among these, 40 proteins were regulated by both TGF-β1 and IL-18 with student's t test difference or –log student's T test < or > 1. We focused on proteins expressed at the cell surface membrane. Few of these molecules were identified and, differently from cytofluorimetric results, in no cases an additive effect of IL-18 was detected. In particular, the protein level of CXCR4 was significantly increased by TGF-β1, but the additive effect of IL-18 was not appreciated. Moreover, no decrements in the protein levels of NKp30 (and NKG2D) were detected in the presence of TGF-β1 used alone or in combination with IL-18. These results showed that sensitivity of the proteomic approach was considerably lower as compared to the flow cytometry.

Nonetheless, we detected a strong, opposite modulatory effect of TGF-β1 and IL-18 on the C-Type Lectin Domain Containing protein 14A (CLEC14A). a molecule involved in cell-to-cell contact and angiogenesis. As shown in Fig 8, its protein level strongly decreased in the presence of TGF-β1, an effect that was completely abrogated by IL-18. Interestingly CLEC14A has been never described in NK cells and no data on its expression and function are available so far.

This and other proteins, that among the 40 selected showed co-regulation by IL-18 and TGF-β1, will be subjected to further analyses.

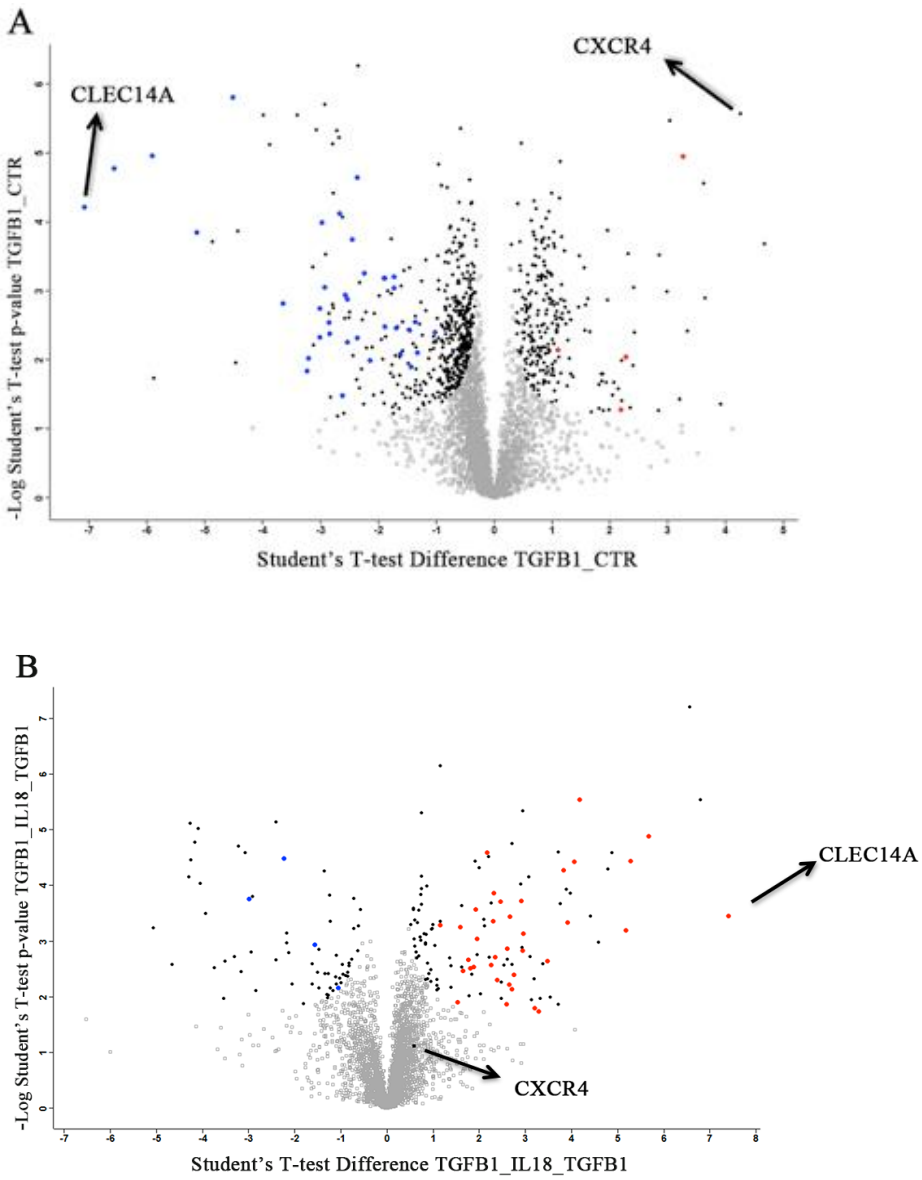

Figure 8. Proteomic analysis of NK cells unconditioned or conditioned with TGF-β1 (TGF-β1 vs CTR data set)

Volcano plot representation of differentially expressed proteins. Plots represent stimulated (TGF-β1) vs. not stimulated (CTR) NK cells **(A)** ot TGF-β1 plus IL-18 vs. TGF-β1 data sets **(B).** Black dots represent proteins that display both large magnitude fold-changes (x-axis, proteins up-regulated after treatment are shown on the right) as well as high statistical significance (FDR = 0.05 and s0 = 0.1 log10 of P value, y-axis). Colored plots represent protein more significantly downregulated (blue) or upregulated by TGF-β1 treatment Gray squares represent proteins not significantly modulated by the treatments. CXCR4 and CLE14A proteins are indicated in both volcano plots.

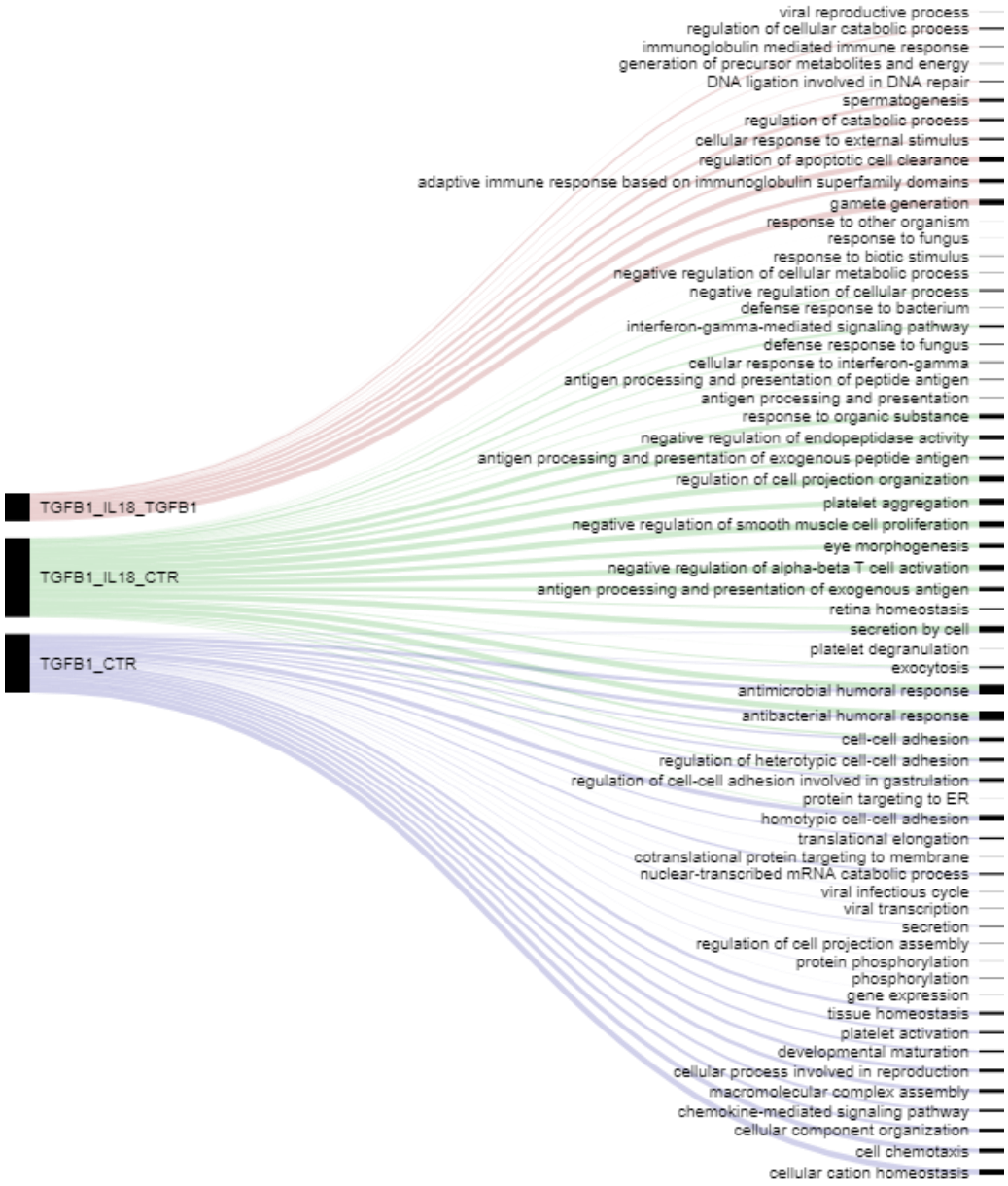

Figure 9. Profile plot of 1D annotation enrichment results.

Enrichment is done by GOBP downloaded from Uniprot with a significance threshold of $p<0.0001$. The output file is converted into a Sankey plot where different colors are associated with different data sets and the width of flow line for each biological process is related to-log10-(p-vaue) of the enrichment.

COMMENT TO THE STUDY AND FUTURE PERSPECTIVE

NK cells play a fundamental role in the immune surveillance of virus infected and tumor cells. However, especially in advanced tumors, the NK cell function is dampened by different escape mechanisms set up by tumor cells or by the other cell types colonizing tumor microenvironment. Mechanisms include the release of soluble factors such as TGF-β1, which in NK cells negatively modulates the expression of crucial activating receptors, reduces IFN-γ production and regulates the migratory properties. TGF-β1 is also fundamental to sustain the expansion of ILCreg, a subpopulation of cells colonizing both mouse and human intestines, which, via IL-10, contribute to the resolution of inflammatory processes [130]. TGF-β1 shapes the differentiation process of ILCs and modulate their effector functions [117]. In particular, it negatively impacts on NK cell differentiation [131] leading to peripheral NK cells with a decidual NK-like phenotype [132] and represents a key mediator regulating both tolerant and regulatory NK cells residing in the liver and in the uterus, respectively [133]. In mouse salivary gland, TGF-β1 is required for the generation of a peculiar ILC subpopulation endowed with both, NK and ILC1 features [132]. In accordance with data showing that TGF-β1 selectively and quickly represses the mTOR pathway [134], a crucial integrator of pro-inflammatory signals, it deeply affects, both in vitro and in vivo, the NK cell functions.

Due to the pleiotropic effect of TGF-β1 on phenotype and functions of NK cells and ILCs, we considered particularly relevant to compare different cytokines for their capability of contrasting TGF-β1 activity. Our study highlighted an unpredictable behavior of IL-18, a cytokine that is listed among the immunostimulatory cytokines. Indeed, we showed that, different from other typical cytokines such as IL-2 or IL-15, IL-18 strengthened rather than weakening some TGF-β1-mediated effects.

IL-18 is produced by macrophages and its role could change depending on the inflammatory context. In acute inflammation such as in response to pathogen-related stimuli, IL-18 produced by M1-polarizing macrophages may synergize with IL-12 and IL-15, thus exerting an immunostimulatory rather that immunoregulatory role. In chronic inflammation processes such as those occurring in tumor tissues, it could support the function of TGF-β1 that is produced/activated by M2-polarized tumor-associated macrophages (TAM). In this context, a membrane-bound form of IL-18 has been described (see Introduction: 2.2 M2 macrophages section) that is expressed by a subset (30-40%) of un-polarized (M0) and M2-polarized macrophages and by the majority of TAM. The mechanisms responsible for its membrane retention and release still remain enigmatic. Interestingly, IL-18 shows many predictable cleavage sites of MMP-2 and -9 (Protease specificity prediction server, PROSPER), extracellular proteases produced by different cell types including M2/TAM that are also involved in TGF-β1 activation. Considering that also tumor cells may represent a source of MMPs, TGF-β1 and IL-18, the synergistic effect of TGF-β1 and IL18 might

be supported by the proteases intervention and play a pivotal role during M2- (and TH2-) polarized immune responses occurring in the tumor microenvironment. According to the IL-18 capability of strengthening the negative regulatory effect of TGF-β1 on tumor immune-surveillance, in several tumor types high concentrations of IL-18 correlated with advanced tumor stages and were significantly, and independently, associated with shorter overall survival [135].

It should also be mentioned that urticaria, asthma, dermatitis, rhinitis, and eosinophilic disorders all have shown a correlation with increased IL-18 levels either in the tissue or in systemically [136].TGF-β1 and IL-18 might also synergize in inducing collagen deposition highly affecting organs' structure and functionality as occurs in tumor-associated fibrogenesis related to radiation therapy, which contributes to morbidity in cancer patients. This synergistic effect might be also crucial in the immune responses against parasites where fibrosis limits their spread through encapsulation. In line with this hypothesis, in mouse model IL-18 has been shown to play a role in the pathogenesis of chronic obstructive pulmonary disease (COPD), the fourth leading cause of death, where tissue fibrosis and collagen deposition represent key pathological features [137].

To conclude, our study highlights an additive effect of IL-18 on some crucial regulatory functions of TGF-β1, strongly supporting the concept that IL-18 can be considered "more than a Th1 cytokine" [138]. First identified as interferon (IFN)-γ inducing factor due to its capability of inducing Type 1 helper T (Th1) cells, IL-18 might cooperate with cytokines exerting opposite, immune-stimulatory (with IL-12, IL-15 or IL-2) or -inhibitory effects (with TGF-β1). The different IL-18 behavior might depend on the relative amount of the various soluble factors characterizing the cytokine milieu, which results from both the type of stress/infectious signal and the different cytokine timing release.

MATERIALS AND METHODS

Cells used in the study

NK cells were purified with "Human NK Cell Isolation kit" (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) from PBMC of healthy volunteer blood donors admitted at the blood transfusion center of IRCCS S. Martino-IST after obtaining informed consent and the study was approved by the Ethics committee of IRCCS S. Martino-IST (39/2012). The degree of purity of the isolated NK cells (CD3-, CD56+, NKp46+) was > 98%.

Monoclonal antibodies and cytokines

The following mAbs were produced in our laboratory: AZ20 (IgG1) (anti-NKp30), BAT221 (IgG1) (anti-NKG2D), MAR93 (IgG1) (anti CD25) and C227 (IgG1) (anti CD69). Anti-human CXCR4 (IgG2b) and anti-human CXCR3 (IgG1) were purchased from R&D Systems (Minneapolis, MN). Anti-human CXCR1 (IgG1) mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human CX_3CR1 PE (rat IgG2b) and the isotype control (rat IgG2b-PE) were purchased from MBL international (Woburn, MA).

Human recombinant cytokines were purchased from PeproTech (rTGF-β1, rIL-15, rIL-12, and rIL-21), Novus Biologicals (rIL-32), Proleukin (rIL-2), MBL International (rIL-18) and R&D Systems (rIL-27). NK cells were cultured for 48 hours in the presence of RPMI 1640 completed medium (supplemented with 2 mM glutamine, 50 mg/ml penicillin, 50mg/ml streptomycin and 5% heat-inactivated FCS) supplemented with the different cytokines used at the following published working concentrations: rIL-12: 1 ng/ml; rIL-15: 20 ng/ml; rIL-18: 100 ng/ml; rIL-21: 20ng/ml; rIL-27: 100 ng/ml; rIL-32 (Dondero et al.)

Flow cytometry

For cytofluorimetric analysis (FACSCalibur Becton Dickinson & Co, Mountain View, CA) cells were stained with the appropriate PE conjugated mAbs or with unconjugated mAbs followed by PE-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associated, Birmingham, AL). Isotype-matched irrelevant mAbs were used as control. On every experimental session, the flow cytometer performances were monitored, and the reproducibility of the fluorescence intensity was aligned by calibrated microsphere (Becton Dickinson & Co, Mountain View, CA).

Real-time PCR

Total RNA was extracted from NK cells using the miRCURY RNA Isolation Kit - Cell and Plant (Exiqon), according to the manufacturer guidelines. 250 ng RNA were reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Real-time PCR was performed using specific TaqMan Gene Expression Assays (Applied Biosystems). CXCR4, NKp30 and CD25 gene expression was normalized to GAPDH gene expression. Experiments were performed in triplicate.

p38MAPK and MEK/ERK inhibitors

Inhibitor SB203580 (p38MAPK) and PD98059 (MEK/ERK) were purchased from Selleckchem. Following recommended procedures both inhibitors were diluted in DMSO (0,1%) and in complete RPMI medium in order to obtain the desired concentrations (20 μM for SB203580 and 80 μM for PD98059). 2x 10^5 resting NK cells were then incubated with p38MAPK inhibitor or MEK/ERK for 1 hour or 72 hours respectively or with DMSO (0,1%) as control. After two washing in complete RPMI medium NK cells were seeded in 96 round bottom wells and conditioned by cytokines for 2 days

Statistical analysis

Statistical analysis with level of significance (p) and graphic representation were performed using Wilcoxon-Mann-Whitney p-value test (non-parametric significance test) and GraphPad Prism 6 (GraphPad Software La Jolla, CA).

AIM2

In recent years great importance has been assumed by immunotherapy as a new strategy to fight cancer, in particular in combination with standard therapies such as chemotherapy and radiotherapy. Along this side the Nobel Prize has been recently assigned to Prof. J.P. Allison and T. Honjo for their discovery on Immune checkpoints limiting anti-tumor immune surveillance. These studies have been fundamental for the development of new combined immunotherapeutic approaches, including the targeting of the PD1/PD-L1 axis. In particular, in metastatic renal cell carcinoma (RCC) the standard therapy, which involved the use of Tyrosine Kinase Inhibitors (TKIs) [139] was strengthened by the use of immune checkpoint blockade (ICB). However, only 20-30% of patients were responders, i.e. showed clinical benefits from ICB (stable disease, partial or complete response). These and other studies highlighted the impact of biological variables on the patients' response to immunotherapy.

Along this line, in recent years several evidences have suggested that microbiome could play a fundamental role in predicting the response to immunotherapy as ICB can affect the integrity of the intestinal barrier [140–142]. Two recent studies by the Zitvogel's group (Routy et al, Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors, Science 2018; Derosa et al., Negative association of antibiotics on clinical activity of immune checkpoint inhibitors in patients with advanced renal cell and non small cell lung cancer, Annals oncology 2018) highlighted that 1) dysregulation of the intestinal microbiome due to antibiotics treatment (ATB) reduced overall survival (Fig.1, left), progression-free survival, and response to immunotherapy of patients with non–small-cell-lung-carcinoma (NSCLC) (Fig.1 left), renal cell carcinoma (RCC), and urothelial carcinoma (UC) (retrospective analysis). Indeed, patients who better responded to immunotherapy (responders) did not receive antibiotics. These observations were confirmed in several cohorts of patients, and validated using a sarcoma mouse model (MCA-205 cell line) (Fig.1 right) showing that tumor-bearing mice treated with antibiotics were significantly less responsive to ICB.

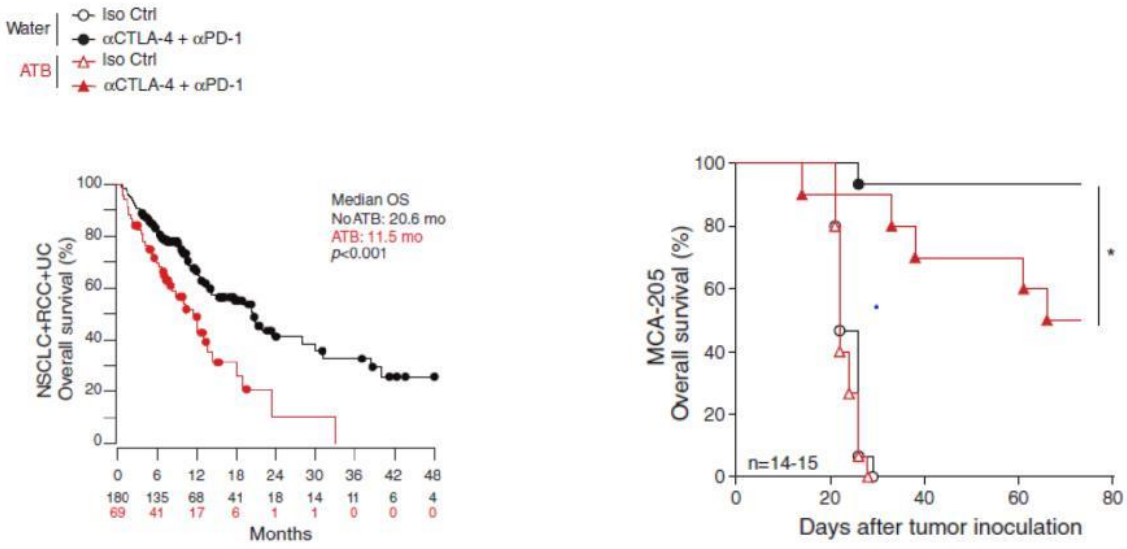

Figure 1. Antibiotics compromise the efficacy of CTLA-4 and PD-1 blockade in cancer patients and in mouse tumor models (Routy et al, Science 2018)

2) feces from NSCLC and RCC patients, responders or non-responders to ICB therapy, showed a different bacterial composition. It has been shown by the use of fecal microbiota transplantation (FMT) using patients' feces to recolonize germ-free or ATB-treated mice in two tumor models (MCA-205 and RENCA). Feces from responder patients conferred to tumor-bearing mice a stronger ICB response as compared to feces from non-responders. Metagenomics analysis of the fecal microbiome showed that the percentage of NSCLC patients (Fig.2) presenting *Akkermansia muciniphila* was significantly higher in responders (PR and SD) as compared to patients with progressive disease (PD). Thus, *Akkermansia muciniphila* was classified as "good" bacterium.

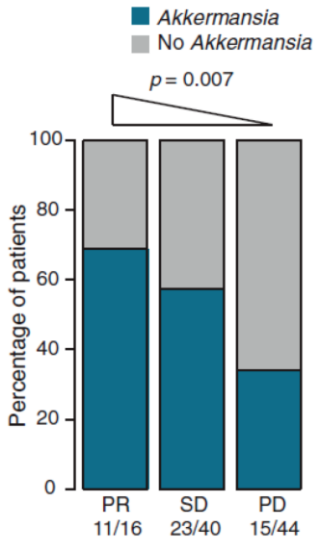

Figure 2. Frequency of patients with detectable *Akkermansia muciniphila* in their feces according to PR (partial response), SD (stable disease), or PD (progressive disease) clinical status. (Routy et al, Science 2018)

3) *Akkermansia muciniphila* was a predictive factor of response to ICB and it was able to contrast resistance to immunotherapy. In particular, in MCA-205 non-responders FMT mice , the oral supplementation with *A. muciniphila* restored the efficacy of anti-PD-1 Ab. A more in-depth analysis suggested some mechanisms responsible for this effect. Indeed, mice recovering response to ICB had dendritic cells capable of producing larger amount of IL-12. Accordingly, mice supplemented by *A. muciniphila* showed a significant increase in the recruitment of $CCR9^{pos}$, $CXCR3^{pos}$ $CD4^{pos}$ T Central Memory (TCM) lymphocytes from the mesenteric lymph nodes to the tumor site (Fig.3 left). Moreover, an increase in the $CD4^{pos}$/FoxP3 ratio in tumor site was observed in mice treated with anti-PD-1 Ab and *A. muciniphila* (Fig.3 right).

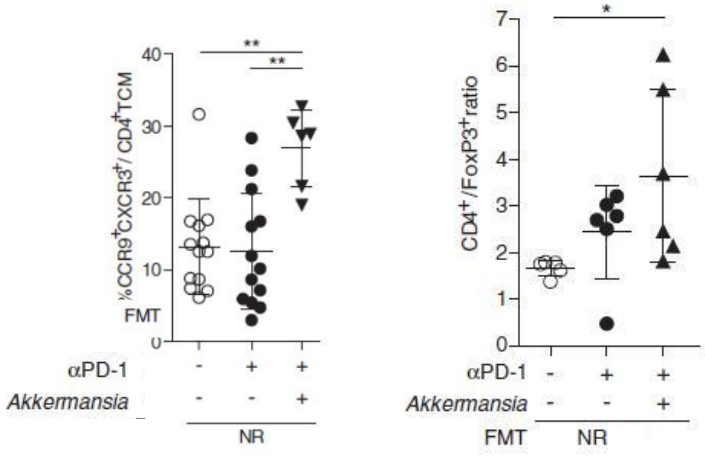

Figure 3. Biological importance of *A. muciniphila* during anticancer PD-1 blockade treatment

Left: flow cytometry analysis of CCR9 and CXCR3 expression in mLN-residing CD4+ TCM among the tumor-infiltrating lymphocytes (TILs) at D+17 after the first injection of PD-1 mAb in ATB-treated animals
Right: Immunohistochemically determination of CD4 and FoxP3 infiltrates in treated tumor sites (Routy et al. Science 2018)

In RCC, a retrospective analysis of responder patients together with a mouse model (RENCA-renal cell carcinoma orthotopically transplanted) showed that responsiveness to ICB therapy was linked to the enrichment of microbiome with *A. muciniphila* and some *Bacteroides spp.* In particular, *Bacteroides salyearsiae, but not Bacteroides xylanosolvens,* was significantly enriched in responder patients (fold ratio of 27.09, *p=0.05).* Accordingly, the resistance to ICB therapy (anti-PD1 + anti-CTLA-4) of mice treated with feces of non-responder patients could be bypassed after the gavage, and consequent intestinal re-colonization, with *A. muciniphila* or *B. salyearsiae* but not with *B. xylanosolvens* (Unpublished Data).

We studied the correlation of the responsiveness to ICB therapy, following microbiome replacement, and changes in the immune compartment in the RENCA mouse model. In particular, we analyzed blood, spleen, mesenteric lymph nodes and tumor site immune cells landscape in non-responder FMT mice and non-responder FMT mice re-colonized with "good" bacteria.

Experimental design:

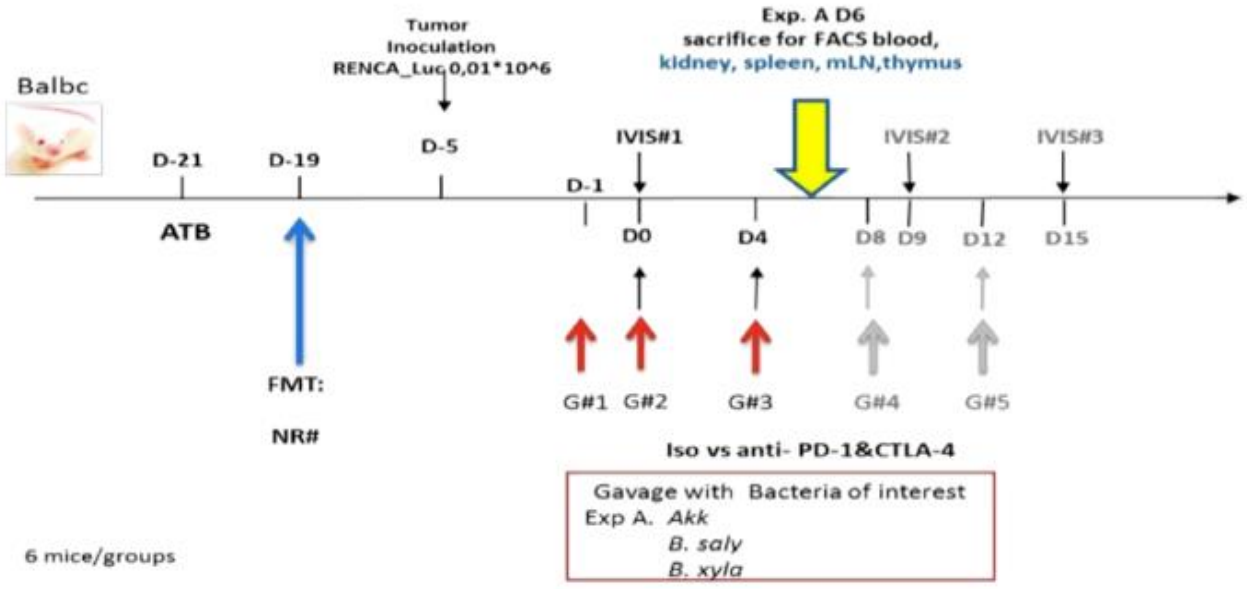

Figure 4: Experimental design

The experimental design is shown in Fig.4 and Table 1.

1) At day -21, BALB/c mice (6 mice/group) were treated with an antibiotic solution (ATB) containing ampicillin (1 mg/ml), streptomycin (5 mg/ml), and colistin (1 mg/ml) (Sigma-Aldrich) added to the sterile drinking water of mice.

2) After 48 hours (day -19), the treatment with antibiotics was stopped and the mice received the FMT with non-responder patients' feces. Frozen fecal samples were thawed and thoroughly vortexed. Large particulate material was allowed to settle by gravity. 200 µl of supernatant was administered in a single dose by oral gavage. An additional 100 µl was topically applied onto the fur of each animal.

3) After 14 days (day -5), mice were anesthetized with isoflurane and 10^4 Luciferase-transfected Renca cell lines, syngeneic for BALB/c mice (kindly provided by Transgene, Illkirch, France) in 30µL PBS were injected into the sub capsular space of the right kidney. Tumor growth was monitored after 5 days using IVIS Imaging System 50 Series (Analytic Jenap) or through the analysis of the weight of kidney.

4) At day -1, the mice received *Akkermansia muciniphila*, *Bacteroides salyearsiae* or *Bacteroirodaes xylanosolvens*. Re-colonization was performed by oral gavage with 100 µl of suspension containing 1×10^8 bacteria (AKK, SALY, XYLA groups).

5) At day 0 and at day 4, mice were treated (intraperitoneal injection) with combined immunotherapy, anti-PD-1 (250µg/mouse; clone RMP1-14,) and anti-CTLA-4 (100µg of anti-

CTLA-4 mAb (clone 9D9) (groups Combo and Combo + bacteria). Control mice received isotype matched Abs (clone 2A3 and MCP11) (group Iso). Moreover, the mice received the oral gavage with bacteria as in day -1. All antibodies were purchased from BioXcell, NH, USA.

MICE GROUPS	ISO	COMBO	*AKKERMANSIA*	*B.SALYERSAE*	*B.XYLANOSOLVENS*
TREATMENTS	ATB FMT-NR Isotypes Abs (2A3 and MPC-11)	ATB FMT-NR ICB (α-PD1+ α-CTLA-4)	ATB FMT-NR ICB (α-PD1+ α-CTLA-4) *Akkermansia muciniphila*	ATB FMT-NR ICB (α-PD1+ α-CTLA-4) *B.salyersae*	ATB FMT-NR ICB (α-PD1+ α-CTLA-4) *B.xylanosolvens*
SAMPLES ANALYZED	Blood Tumor site (kidney) Spleen Mesentheric lymphonodes	Blood Tumor site (kidney) Spleen Mesentheric lymphonodes	Blood Tumor site (kidney) Spleen Mesentheric lymphonodes	Blood Tumor site (kidney) Spleen Mesentheric lymphonodes	Blood Tumor site (kidney) Spleen Mesentheric lymphonodes

Table 1: mice groups, treatments and sample analyzed

Samples collection and flow cytometry analysis

Tumors, mesenteric lymph nodes (mLN), blood and spleens were harvested at day 6 (i.e 7 days after the first bacterial gavage and 6 days after the first injection of anti-PD-1 and anti-CTLA-4 mAb).

During blood sample collection, animal will be in terminal anesthesia. 0.3 - 1 ml of blood can be obtained depending on the size of the mouse from the heart, preferably the ventricle, which can be accessed either via the left side of the chest, through the diaphragm, from the top of the sternum or by performing a thoracotomy. Blood should be withdrawn slowly to prevent the heart collapsing. Excised tumors were cut into small pieces and digested in RPMI medium containing Liberase (25 µg/mL) and DNase1 (50 UI/mL) for 30 minutes at 37°C, crushed and filtered twice using 100 and 70 µm cell strainers. Lymph nodes and spleen were crushed in RPMI medium and subsequently filtered through a 100-µm cell strainer.

The cells obtained were counted with Vi-CELL Cell Viability Analyzer (Beckman Coulter), stained with direct fluorescent antibodies (BD) and analyzed by flow cytometry. To detect NKp46+ cells, including NK cell population, an anti-mouse antibody specific for NKp46 molecule (29A-1.4, BD), has been used.

PRELIMINARY RESULTS:

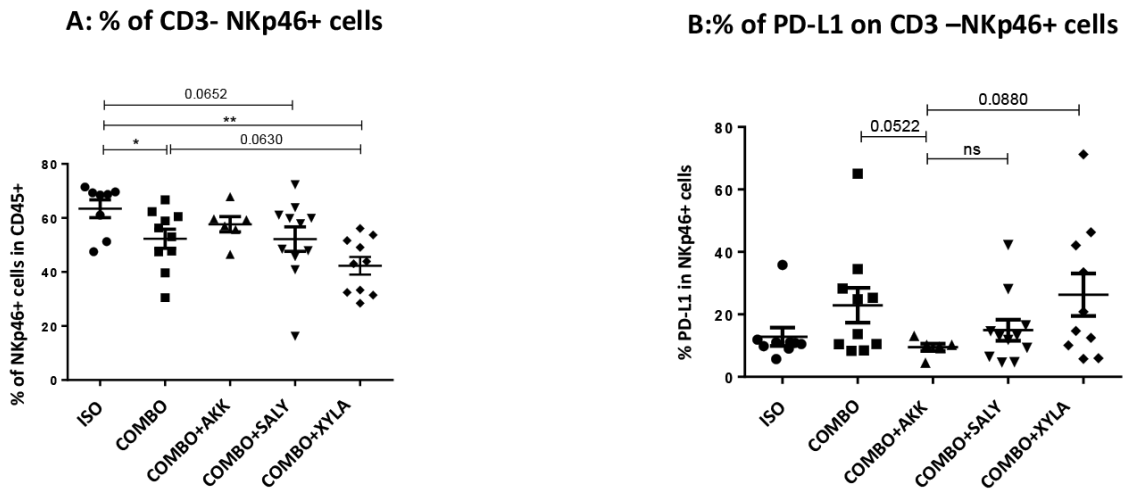

Figure 5: Analysys of % of NKp46+ cells (A) and PD-L1+NKp46+ cells (B) in peripheral blood

	Combo vs Iso	Combo+Akk Vs Combo	Combo+Saly Vs Combo	Combo+Xyla Vs Combo
NKp46+ cells	REDUCED	=	=	=
% PD-L1+ NKp46+ cells	=	REDUCED	=	=

Table 2: Comparison of the different conditions regarding the % of NKp46+ cells and of PD-L1+ NKp46+ cells

In peripheral blood, the percentage of CD3- NKp46+ cells was significantly lower in Combo mice as compared to Iso (Fig5A and Table 2). On the contrary no significant variations in NKp46+ cells were observed in COMBO + bacteria mice groups as compared to Combo mice group.

In COMBO mice NKp46+ cells also show a clear tendency to increase the percentage of PD-L1 molecules (Fig-5B).This effect was influenced by the recolonization with *Akkermansia muciniphila* but not with other bacteria. Indeed, mice group recolonized with "the good bacteria" did not show significant increase in the percentage of NKp46+ PD-L1+ cells.

In spleen and secondary lymphoid organs (Fig.6), the percentage of CD3- NKp46+ cells was not altered by the treatment with ICB, as compared to ISO. However, in the spleen the percentage of CD3-NKp46+ was significantly higher in Combo mice re-colonized with *Akkermansia muciniphila* and even more evident in those that received *Bacteroides salyearsiae*. The treatment

with *Bacteroirodaes xylanosolvens* didn't affect the percentage of CD3- NKp46+ in spleen, whereas it was associated with a strong increase of their number in mesenteric lymph nodes.

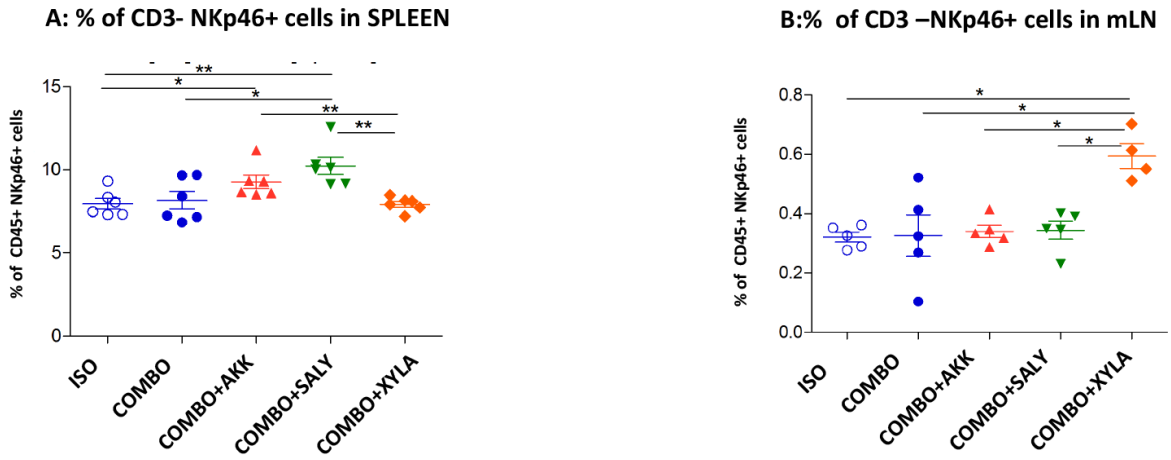

Figure 6: Analysys of % of NKp46+ cells in spleen (A) and in mesentheric lymphnodes (B)

Finally, we analyzed CD3-NKp46+ cells in tumor site (Fig.7). Although a statistic significance was not reached, the analysis showed that the % CD3-NKp46+ cells tends to decrease in ICB treated mice that have received FMT from NR patient (not to be expected to be responsive to therapy). Combo mice receiving "good" bacteria showed a trend in restoring the percentage of CD3-NKp46+ cells.

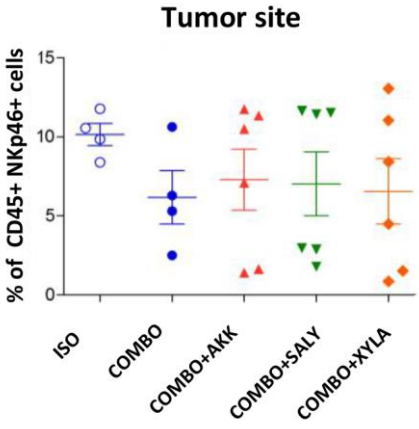

Figure 7: Analysys of % of NKp46+ cells in tumor site (kidney)

COMMENT TO THE STUDY AND FUTURE PERSPECTIVE

Our preliminary results indicate that the combined ICB treatment in mice FMT from non-responder patients results in a significant reduction of circulating NKp46+ cells. This appears to involve other immune cells including CD8+ T cell population (data not shown). Two hypotheses may explain the results: a) ICB treatment may affect the viability of NKp46+ cells; b) ICB treatment might influence the exit of NK cells from the blood and their recruitment into peripheral tissues. CD3- NKp46+ cells also show enrichment in PD-L1+ cells, a phenomenon that seems to be counteracted by re-colonization with *Akkermansia muciniphila, "*the good bacteria". Further analyses are necessary to confirm these data and to evaluate in additional tumor model, if this phenomenon is tumor-specific. The decreased number of PD-L1+ NKp46+ cells observed in a favorable background (i.e. in the presence of *Akkermansia muciniphila)* might positively influence the responses to ICB. Indeed, PD-L1 on NK cells may negatively regulate the function of immune cells types expressing the specific receptor (PD-1 or CD80) such as NK cells themselves, T cells and macrophages. Along this line, ICB treatment in a favorable background led to a higher percentage of NKp46+ cells in the spleen, but not in mesenteric lymph nodes. Future analysis will analyze PD-L1 expression in both spleen and mLN. An interesting hypothesis may be that spleen, rather than mLN, represents crucial site for the orchestration of ICB-based anti-tumor responses. Regarding the tumor site, the percentage of NKp46+ cells seems to decrease in COMBO mice and to increase upon re-colonization with good bacteria. In order to confirm and to strength these data, further analysis will be done at later time points. Moreover, in-vivo depletion of NKp46+ cells could be used to demonstrate the effective relevance of NKp46+ populations in the outcome of ICB-treatment.

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Review

1) Overview of the soluble and membrane-bound tumor factors limiting NK-mediated immune surveillance.

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2) Main NK cell receptors and their ligands: regulation by microRNAs

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3) Molecular mechanisms directing migration and retention of Natural Killer cells in human tissues

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4) Imatinib and Nilotinib Off-Target Effects on Human NK Cells, Monocytes, and M2 Macrophages.

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Review Article OMICS International

Overview of the Soluble and Membrane-bound Tumor Factors Limiting NK-mediated Immune Surveillance

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Abstract

Many evidences suggest that NK cells are effective in patrolling for and eliminating tumors in their onset phase, but hardly limit the progression of large established solid tumors. Beside the transition of tumor cells towards a more aggressive phenotype, the NK cell efficacy might be limited by a complex immunosuppressive milieu present in the tumor microenvironment. Indeed, different mechanisms damping NK cell function have been shown in these last years. These include a plethora of tumor-derived immunomodulatory soluble factors (TGF-β, MIF, adenosine, L-Kynurenin, PGE2) as well as soluble ligands (MICA, ULBP-2, PVR, B7-H6) that compete with membrane-bound tumor ligands for binding to activating NK receptors. During NK-tumor cell contact the NK cell function can also be inhibited by the engagement on NK cells of different inhibitory receptors. The specific ligands might be either constitutively expressed at the tumor cell surface (HLA-I, B7-H3, PVR) or de novo induced/up-regulated (PD-Ls) by immunostimulatory factors (IFN-γ, TNF-α). These are largely released during the active phases of the immune responses and exert an unwanted side effect called "tumor adaptive immune resistance". This review aims to summarize the best-known molecular mechanisms that, at various times and in different ways, can limit the efficacy of the NK-mediated immune surveillance of tumors.

Keywords: NK cells; Tumor microenvironment; Soluble factors; TGF-β1; HLA-I; Immune-checkpoints; PD-1/PD-L1; B7-H3

INTRODUCTION

Natural killer cells (NK) are crucial cytolytic effectors belonging to the family of innate lymphoid cell (ILC) [1,2]. Originally described as cells exerting a "natural" cytolytic activity due to their capability to kill the highly susceptible K562 eritroleukemia cell line, it is now well established that they require activation to exert optimal effector functions. Moreover, the susceptibility to NK-mediated killing of established tumor cell lines is superior to that of tumors *ex-vivo* isolated from patients, as occurs in bone marrow metastases that are much more resistant to NK-mediated aggression [3]. This supports the concept that an effective NK-mediated anti-cancer activity can't dispense with an optimal activation of endogenous or adoptively transferred NK cells. It is also crucial to understand which NK cell subset, once activated, can exert the most effective anti-cancer activity in a particular immunotherapeutic setting. Indeed, it has been recently stressed that a great heterogeneity in NK cell phenotype and functions exists that goes beyond the classical $CD56^{dim}$ $CD16^{high}$ and $CD56^{bright}$ $CD16^{low/neg}$ NK cell dichotomy [4]. Beside the identification and description of $CD56^{neg}$ NK cells [5] that are particularly abundant in peripheral blood of virus-infected donors, several studies highlighted the great heterogeneity of peripheral blood $CD56^{dim}$ NK cells, which include subpopulations characterized by different capabilities of being activated by cytokines, antibodies or tumor contact [6,7]. Thus, adoptive transferred NK cell-based therapeutic protocols should combine optimal activation strategies, the selection of the best NK cell subpopulation, consider the *in vivo* persistence of the *in vitro* activated NK cells, and, last but not least, their chemokine receptor repertoire. Indeed, the scarce attitude of cytotoxic $CD56^{dim}$ NK cells to reach and invade the tumor parenchyma represents a major obstacle for the effectiveness of NK cells, especially in the therapy of solid tumors [8,9]. This might occur also in a chemokine-rich tumor milieu, due to a defective expression in NK cells of the chemokine receptors involved in their migration toward peripheral tissues. This hypothesis matches with the scenario described in neuroblastoma patients whose peripheral blood CD56dim NK cells are characterized by an unusual reduced expression of CX3CR1, the fractalkine (CX3CL1) receptor [10]. Interestingly, CX3CR1 has been shown to be deeply down-regulated by transforming growth factor beta 1 (TGF-β1), a pleiotropic soluble factor released by most cell types including tumors that is capable of modulating pivotal effector functions of different immune cells [11,12]. TGF-β1 is one among the several soluble factors present in the tumor microenvironment that recently emerged as potent immune-modulators. Moreover, beside the classical HLA class I-mediated inhibition of the NK cell activity, several additional inhibitory signals have been recently described that, during NK-to-tumor contacts, limit the NK-mediated immune-surveillance. Thus, the choice of the most effective, activated NK cell subsets should not disregard their profile in terms of the cognate inhibitory receptor-ligand interactions. This review summarizes the best-known tumor-derived soluble factors and tumor-associated surface molecules exerting an immunomodulatory role in NK cells.

Soluble Immunomodulatory Mediators

MIF, adenosine, L-Kynurenin, PGE2

In the past few years, many different tumor-associated regulatory/suppressive mechanisms have been widely studied (Figure 1). Among these, molecules shed from the tumor cell surface, such as the soluble ligands of NKG2D [13-15], DNAM-1 [16] and NKp30 [17], activating receptors crucial for T- and/or NK cell-mediated immune-surveillance [18]. sMICA, sULBP-2, sPVR and sB7-H6 compete for binding with the ligands expressed on the tumor cell surface, thus hindering the efficient target recognition. Moreover, different tumor-secreted soluble mediators have been shown to clearly suppress the efficacy of the immune system. Among these, the macrophage migration inhibitory factor (MIF, also known as Glycosylation-Inhibiting Factor) [19-21] and adenosine, an endogenous purine nucleoside highly produced by tumors expressing CD39 and CD73, ectonucleotidases converting ATP to adenosine. Both MIF and adenosine have been demonstrated to inhibit cytotoxicity and cytokine production in human NK cells, the second mainly through the engagement of the adenosine receptor 2A (AdoR2A), which is coupled to adenylyl cyclase *via* Gs protein [22,23]. In the mouse model the use of AdoR2A antagonist reduced the metastatic potential of CD73+ tumors [24] that was further reduced by blockade of AdoR2B. Since *in vitro* AdoR2B blockade had no significant effects on NK cell cytotoxicity, the benefit observed in tumor-injected mice treated with AdoR2 antagonist might depend on both NK cell-dependent and -independent mechanisms. Along this line, in mice selectively lacking AdoR2 expression in myeloid compartment it has been shown that adenosine can indirectly suppresses T and NK cell-mediated antitumor activity by shaping the functions of different myeloid cells. In particular, in this mouse model, a reduced melanoma growth was associated with a significant increase in MHC class II expression and IL-12 release in tumor-associated macrophages (TAM), features fitting with an M1-like pro-inflammatory macrophage polarization. Moreover, AdoR2neg TAM, dendritic cells (DC), and myeloid-derived suppressor cells (MDSC) showed a clear reduction in IL-10 expression, a cytokine originally described as a negative regulator of IL-12 production in LPS-stimulated peripheral blood mononuclear cells [25]. To date IL-10 cannot be definitively included among the tumor-derived cytokines limiting NK cell activity since both stimulatory and inhibitory effects on NK cell functions have been described. On the contrary, a huge number of data have demonstrated the immunomodulatory function of L-kynurenine, the tryptophan catabolite derived from indoleamine 2,3-dioxigenase 1 (IDO1) pathway [26], and of Prostaglandin E2 (PGE2). Both factors deeply affect the cytokine-mediated upregulation of the expression and function of different activating NK receptors such as NKp46, NKp44 and NKG2D [27-29] a mechanism that seems to involve the c-Jun N-terminal Kinase (JNK) pathway [29]. The extent of *in vivo* suppression mediated by L-kynurenine and PGE2 might be considerable since these factors are released by several cell types colonizing tumor microenvironment, including the cancer-associated fibroblasts (CAF) [30,31], MDSC and DC [32]. In particular, IDO-expressing DC exert a deep immune-suppressive effect by affecting not only proliferation and effector function of NK cells, but also by inducing the conversion of CD4+ T cells into CD4+ CD25+ Foxp3+ regulatory T cell (Treg) [33]. Tumor-derived PGE2, *via* the EPA4 receptor [34,35] decreases human NK cells proliferation, granzyme B/perforin content [36] and drives NK cells towards apoptosis [37]. Moreover, *via* the EPA2 and EPA4 receptors, PGE2 induces the release of TGF-β1 by MDSC [38] that further inhibit NK cell activity. TGF-β1 represents a secretory immune-suppressive hallmark of several other cells in tumor microenvironment including Treg and TAM [39].

TGF-β1

TGF-β1 is the prototypic tumor-derived immunomodulatory soluble mediator, although many additional functions have been described over the years, highlighting its pleiotropic activity. Different studies reported a significant contribution of TGF-β1 in the epithelial to mesenchymal transition (EMT), a process that allows tumors of epithelial origin to acquire a less differentiated, invasive and pro-metastatic phenotype [40,41]. TGF-β1 has been shown to suppress the differentiation process and the effector functions of several immune cells [12]. In particular, it represses the development of human NK cells from CD34+ progenitors and inhibits differentiation of $CD16^{pos}$ NK cells [42], TGF-β1 promotes the conversion of peripheral NK cells to a decidual NK-like phenotype [43] and, as shown in mouse salivary gland, it drives the differentiation of a particular ILC subpopulation sharing NK and ILC1 features [43]. Released as a large latent complex, TGF-β1 remains biologically unavailable until its activation in inflammatory sites such as the tumor microenvironment by signals including low pH, heat, proteases and members of the integrin receptor family [11]. Once activated, TGF-β1 shows a potent inhibitory effect on NK cells, both *in vitro* and *in vivo*, limiting the main NK cell effector functions including IFN-γ production and cytotoxicity [44-48]. In this context, *in vitro* conditioning of NK cells with recombinant TGF-β1 (rTGF-β1) caused severe downregulation of the surface expression of NKp30 and NKG2D, activating NK receptors cooperating in recognition and killing of several tumor histotypes [44]. The same occurred with rTGF-β2 [10], although knockout mice lacking TGF-β1 or TGF-β2 showed distinct phenotypic features suggesting that the two isoforms could also have specific, non-overlapping functions [49]. Interestingly, while the TGF-β1-mediated downregulation of NKp30 occurred at the transcriptional levels no significant changes in NKG2D transcript was observed in TGF-β1-treated NK cells [44]. Accordingly, it has been shown that the reduced NKG2D surface expression observed in the presence of TGF-β1 both *in vitro* and *in vivo*, is due to its capability of downregulating at transcriptional and translational level DAP10, the signaling subunit associated with NKG2D [50-52]. Very recently it has been shown that TGF-β1 also inhibits the IL-15-induced NK cell activation, particularly by selectively and quickly repressing the mTOR pathway [53], a crucial integrator of both pro- and anti-inflammatory signals. Recent data also suggest that the tumor-derived TGF-β1 might modify the migratory capability of NK cells. Indeed, it has been shown that neuroblastoma (NB) cell lines spontaneously release amounts of TGF-β1 capable of modulating the chemokine receptor repertoire of NK cells [10]. In particular NB-derived TGF-β1 increases CXCR4 and CXCR3 surface expression in all NK cells whereas it decreases that of CX3CR1 in the $CD56^{dim}$ NK cell subset. Notably, unusual $CX3CR1^{low}$ $CD56^{dim}$ and $CXCR3^{high}$ $CD56^{bright}$ NK cell populations were observed in peripheral blood of patients with high risk NB (stage 4 or M) [10]. Thus, tumor-derived TGF-β1 can affect the expression of chemokine receptors that play a key role in the bone marrow homing, egress, interaction with endothelium and recruitment into peripheral tissues of NK cells. Recently, in a mouse model of pulmonary allergic responses, it has been shown that TGF-β1 is crucial for the generation of allergic response acting as chemotactic factor recruiting ILC2 and eosinophils [54]. Importantly, it has been demonstrated that TGF-β1 modulates in tumor cells, the expression of specific microRNAs and up-regulates B7-H3 (CD276) [55], a molecule belonging to the family of immune

checkpoint proteins, which acts as co-inhibitory factor and limits the function of NK cells. Overall data suggest that TGF-β1 antagonists, capable of overcoming or blocking its immunomodulatory effect might represent a valuable adjuvant therapy in the cure of different tumors. In this context, it has been recently shown that blocking of TGF-β1R in combination with antibodies targeting the NB-associated antigen GD2, potentiates the NK-mediated anti-NB activity leading to a reduced tumor growth and increased survival of mice injected with NB cell lines or patient-derived neuroblasts [56]. Moreover, ongoing clinical trials will evaluate the benefit of TGF-β or TGF-βR blockade in neoplastic patients (ClinicalTrials.gov NCT02452008; NCT02581787). It is of note however that, due to the wide homeostatic regulatory role of TGF-β, in order to obtain specific and restrained therapeutic effects, light should be made on the signaling pathways mediated by the different TGF-β isoforms and on the mechanisms regulating the immunomodulatory effects.

NK-To-Tumor Contact Immunomodulatory Signals

KIRs/HLA-I and NKG2A/HLA-E

The most powerful inhibitory pathway affecting the NK cell activity is represented by the interaction between HLA class I molecules (HLA-I) on target and specific inhibitory receptors on NK cells (Figure 1). These inhibitory receptors include Killer Ig-like Receptors (iKIRs), clonally distributed receptors distinguishing among allotypic determinants of the classical HLA-A, -B and - C, and the CD94/NKG2A heterodimer specific for HLA-E [57-59]. Mature NK cells can also express the activating counterpart of these HLA-I specific inhibitory receptors, i.e. activating KIRs (aKIRs) and CD94/NKG2C [60,61]. During NK cell maturation the engagement of inhibitory receptors by their self HLA-I ligands confers functional competence to NK cells, through a process referred to as "licensing" or "education" that has been explained by different models [62-64]. Based on the "rheostat" model, during NK cell education NK cell reactivity is tuned by the strength of the inhibitory signal induced by self-HLA-I molecules [65]. The inhibitory receptor repertoire acquired by NK cells during maturation guarantees that in normal conditions inhibitory signals prevail on the activating ones safeguarding HLA-I^{+} autologous healthy cells from NK cell-mediated killing. The NK cytolytic activity is unleashed in pathologic conditions such as virus infection or tumors where transformed cells increase the expression of ligands for activating NK receptors while downregulating that of HLA-I [66]. NK cell reactivity can be limited when tumor cells retain high levels of HLA-I expression, as occur in hematological malignancies such as acute lymphoblastic leukemia (ALL). However, it has been shown that in allogeneic settings such as in the context of haploidentical hematopoietic stem cell transplantation (haplo-HSCT), the differentiation of alloreactive NK cells from the donor, i.e cells expressing KIRs specific for HLA-I molecules absent in the recipient, strongly improve anti-leukemic surveillance [67,68].

The studies describing the beneficial graft versus leukemia (GvL) effect of alloreactive NK cells in haplo-HSCT has inspired the design of new immunotherapies aimed to enhance anti-tumor NK cell reactivity by blocking the interactions between HLA-I and iKIR or CD94/NKG2A. A fully human anti-KIR mAb (1-7F9, Lirilumab, IPH2101) that recognizes iKIRs (KIR2DL1, KIRDL2 and KIR2DL3) has been generated [69], which favors the NK cell-mediated killing of HLA-matched tumor cells as documented *in vitro* and *in vivo* in phase I/II clinical trials involving Acute Myeloid Leukemia (AML) and multiple myeloma (MM) patients, [69,70]. Interestingly, Lirilumab has been successfully combined with the anti-CD20 rituximab to augment NK-mediated cytotoxicity against lymphoma cells *in vitro* [71]. Based on the broad expression of HLA-E on both solid and hematological malignances, a novel therapeutic approach has been designed to block the CD94/NKG2A-HLA-E interaction, by using the humanized anti-NKG2A Monalizumab, which is currently in a phase I/II clinical trial [72].

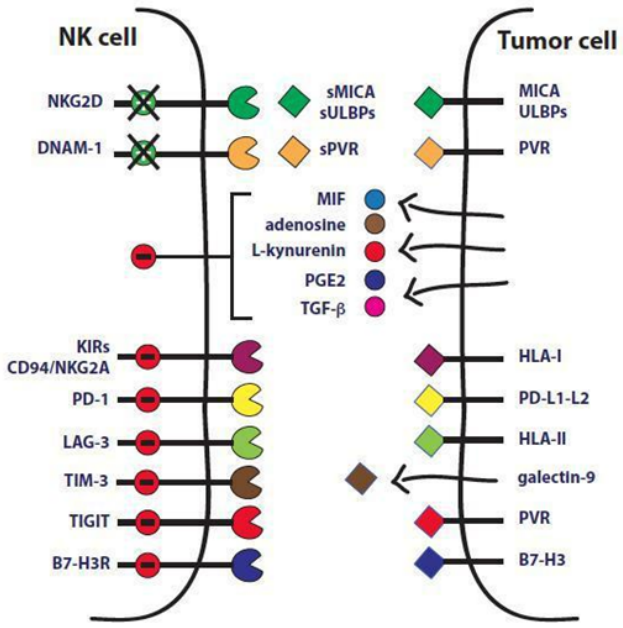

Figure 1: Soluble and membrane-bound factors dampening NK-mediated anti-tumor activity.

This novel approach was developed upon the observation that NKG2A+ NK cells predominate in the early period of immune reconstitution after HSCT, thus representing optimal targets to potentiate NK cell-mediated anti-leukemic activity [73]. In addition, NKG2A+ NK cells express higher levels of activating receptors such as NCRs as compared to more differentiated KIR+ NKG2A- NK cells [6]. The high expression level could compensate the low cytotoxic potential displayed by these less differentiated NK cells. KIRs, CD57 and LIR represent phenotypic hallmarks of terminally differentiated NK cells, which show a good cytolytic potential but poor responsiveness to cytokines [74]. Interestingly, in individuals exposed to pathogens such as cytomegalovirus (CMV), the mature NK cell population is characterized by a very high percentage of cells expressing CD94/NKG2C or aKIR [75-78], which show features similar to cells of adaptive immunity including clonal expansion capability, strong effector functions and longevity. This "memory-like" population represents a powerful candidate for adoptive NK cell transfer therapy in cancer patients [79]. Along this line it is crucial to define whether its efficacy might be limited during NK-to-tumor contacts by additional inhibitory or co-inhibitory signals, whose activity might be particularly relevant in the context of HLA-I^{low} or negative tumors.

PD-1/PD-Ls, LAG-3/HLA-II and TIGIT/PVR

The PD-1/PD-Ls axis is a well-known immune checkpoint, i.e. inhibitory pathways that physiologically maintain self-tolerance and limit the duration and amplitude of T cell immune responses, thus minimizing tissue damage [80-82]. The PD-1 receptor (CD279) has been demonstrated to limit T proliferation and switch off the T cell functions mostly in peripheral tissues. More recent reports show the presence of PD-1^{pos} NK cells in both cancer patients and in a relevant proportion of healthy donors who were serologically positive for human CMV [83-86]. PD-1 expression is confined to terminally differentiated NKG2A^{neg}KIR^{pos}CD57^{pos} NK cells and their antitumor activity can be partially restored *in vitro* by antibodies disrupting the interaction between PD-1 and its cellular ligands PD-L1 and PD-L2 [86,87]. PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) belong to the B7 family that consists of several members including B7-H3 (see below) (Figure 1). PD-L1 is expressed in several normal tissues, whereas PD-L2 is mainly restricted to antigen presenting cells (APC) [80,81]. Importantly, both inhibitory ligands can be expressed by tumor cells in response to immunostimulatory factors such as IFN-γ and TNFα that are released by activated T and NK cells [80,85,88]. This phenomenon called "adaptive immune resistance" [88] is a mechanism of escape by which cancer cells adapt their phenotype to the pressure of immune responses. Interestingly, it has recently been shown that PD-L1^{neg} metastatic cells purified from bone marrow aspirates of high risk NB patients have different capabilities of up-regulating PD-L1 in response to IFN-γ [85]. The lack of PD-Ls upregulation in some patient might contribute to a reduced clinical response to therapy with anti-PD-1. However, it should not be disregarded that the therapeutic efficacy of anti-PD1 antibodies has been observed also in patients who at the time of therapeutic decision carried PD-L1^{neg} tumors. Indeed, the clinical benefit might also depend on the strengthening of the crosstalk between PD-Ls+ APC and PD-1+ immune effectors including T or NK cells. IFN-γ is also a potent inducer of HLA class II molecules that can be recognized by LAG-3, an additional inhibitory mechanism that recently emerged in human NK cells together with TIGIT/PVR and TIM-3/galectin-9 interactions (Figure 1) [89-92]. Interestingly, the heterogeneous expression of the TIGIT inhibitory receptor observed in NK cells from healthy individuals inversely correlates with their capability of performing degranulation and IFN-γ release in response to IL-12 stimulation [91]. Moreover, low TIGIT expression has been described in CMV-induced terminally differentiated NK cells that appear more resistant to the inhibitory effect mediated by PVR^{pos} MDSC. Since studies explored the cytokine-induced expression of these co-inhibitory receptors mainly in long term-cultured NK cell lines such as NK92 [93], data on primary NK cells are required to better understand the relative contribution of these inhibitory pathways and the kinetic that regulates their emergence.

B7-H3R/B7-H3

Another interesting NK-to-tumor contact inhibitory pathway is mediated by the B7-H3 ligand, a tumor-associated surface molecule, also present in tumor-derived exosomes [94], which is endowed with both immune-regulatory and pro-tumoral functions. B7-H3 is capable of inhibiting the cytolytic activity of human NK cells against neuroblasts purified from bone marrow aspirates of high risk NB patients, which are characterized by reduced levels of HLA-I [18] and adhesion molecules (unpublished observation). The B7-H3-mediated inhibitory effect, which depends on its interaction with a still unknown inhibitory receptor, is particularly evident when using xenogenic B7-H3^{high} transfectants [95]. This suggests that this inhibitory pathway might require peculiar conditions to be unleashed, which could be represented by poor engagement of potent inhibitory NK receptors and/or by the presence of weak activating signals. It should be mentioned that in mouse B7-H3 has been described as a "friend" in tumor immunology [96]. In particular, intratumoral injection of an expression plasmid encoding mouse B7-H3 led to a complete NK- (and T-) mediated regression in approximately half of tumor-bearing mice [97]. Interestingly, the mouse *B7-H3* gene codes for a molecule characterized by two Ig-like domains while human cell tissues predominantly express a four Ig-like domains isoform resulting from exon duplication [98]. While in human B7-H3 is still an orphan ligand, an activating receptor has been identified in mice that is represented by TREM-like transcript 2 (TREML2, TLT-2), expressed by activated T cells and myeloid cells [99]. Importantly however, Leitner and co-workers who extensively faced this issue did not find evidence for B7-H3/TREML2 interaction in human [100]. Considering that B7-H3 belongs to the B7 family that includes members interacting with both activating and inhibitory receptors, it can't be excluded the existence of a complex scenario resulting from the capability of B7-H3 to engage receptors with opposite signal. However, to date most *in vitro* and *in vivo* data lean toward a B7-H3 inhibitory role in human and the B7-H3R/B7-H3 axis has been included among the immune checkpoints [81,101,102]. An adjuvant therapeutic strategy in cancer might be represented by antibodies disrupting the interaction between B7-H3 and its receptor/s. Different phase I Clinical trials are ongoing with humanized anti-B7-H3 mAbs (NCT02628535; NCT02982941; NCT02475213) [81] and encouraging results have been obtained in the first in-human intrathecal injection of radioiodinated anti-B7-H3 Ab (following standard therapy) in 21 neuroblastoma patients with recurrent Central nervous system (CNS) metastasis [103]. It is of note that the therapeutic efficacy of anti-B7-H3 mAbs might depend not only by the strengthening of the NK- (and T-) mediated anti-tumor responses, but also by the weakening of the direct pro-tumoral activity of B7-H3. Indeed, studies in tumor of different histotype showed that high B7-H3 expression drives tumor cell progression through different molecular mechanisms. These include promotion of migration and invasiveness [104] and reduction of sensibility to chemotherapy-induced apoptosis, as demonstrated in breast [105] and pancreatic carcinoma [106]. Accordingly, high expression of B7-H3 is a negative prognostic factor in several tumors including neuroblastoma [18,107-111]. In particular, in primary neuroblastoma high B7-H3 surface expression, in terms of both intensity and percentage of positive cells, has been correlated with poor event-free survival also in patients with localized disease (stage 1–3), suggesting that high B7-H3 expression might discriminate between low- and high-risk patients who need a more careful follow-up.

Conclusions and Future Perspective

NK cell-based immunotherapy is becoming a promising approach for the treatment of both hematological malignances and solid tumors. However, recent published data show that the complexity of the immune-suppressive milieu characterizing the tumor microenvironment can't be neglected. Indeed, different inhibitory mechanisms represented by soluble factors or by tumor-associated surface ligands could deeply reduce the NK cell activity against tumors. Importantly, malignant cells can constitutively express some of these ligands (HLA-I, B7-H3) or increase/de novo induce their expression (PD-Ls) as an adaptive defense mechanism promoted by

immunostimulatory factors (IFN-γ) that are released during effective NK and TH1 cell-mediated immune responses. Thus, future immunotherapeutic interventions should consider the possible onset in patients of multiple, different immunosuppressive mechanisms affecting the function of endogenous or adoptively transferred NK cells. Also the chemokine receptor repertoire acquired by NK cells during their *in vitro*-expansion needed for the adoptive transfer in patients should not be neglected. Along this line, it might be relevant to hinder the effects of factors (TGF-β1) capable of modifying, in endogenous or infused NK cells, the expression of chemokine receptors crucial for their extravasation and recruitment at the tumor sites.

Executive summary

NK cells and cancer immunotherapy

NK cells are crucial cytolytic effectors in anti-tumor immune responses.

NK cell-based immunotherapy is becoming a promising approach for the treatment of both hematological malignances and solid tumors.

Ready to kill or killers who require to be armed?

Originally described as cells exerting a "natural" cytolytic activity it is now well established that, to exert optimal effector functions, NK cells require activation *via* immunostimulatory cytokines and tumor contact.

NK cell population is heterogeneous and includes subsets characterized by different capabilities of being activated and different effector functions.

Once activated, NK cells need a chemokine receptor repertoire ideal to their recruitment in inflamed tissues such as tumors.

How tumors may dump NK cell function *in vivo*?

Tumor cells purified from patients are less susceptible to NK-mediated killing than established tumor cell lines commonly used *in vitro*.

A complex immunosuppressive milieu is present in the tumor microenvironment.

A plethora of tumor-derived immunomodulatory factors exists, either soluble or membrane bound, that might limit the NK-mediated immune-surveillance *in vivo*.

Soluble immunomodulatory mediators

Tumor cells may release soluble ligands (sMICA, sULBP-2, sPVR and sB7-H6) that compete with membrane-bound tumor isoforms for binding to activating NK receptors.

Different tumor-secreted soluble mediators (MIF, adenosine, L-kynurenine, PGE2, TGF-β1) have been demonstrated to inhibit NK cell function including proliferation, cytotoxicity and cytokine production. These factors can be released by tumor cells as well as by other cell types colonizing the tumor microenvironment (TAM, DC, MDSC, CAF).

TGF-β1 is the prototypic tumor-derived immunomodulatory soluble mediator. In NK cells, it represses development from CD34+ progenitors and differentiation, limits the main effector functions (IFN-γ production and cytotoxicity), reduces the expression level of activating receptors (NKp30, NKG2D) and alters the chemokine receptor repertoire.

NK-to-tumor contact immunomodulatory signals

NK cell reactivity can be limited when tumor cells retain high levels of HLA-I expression, as occur in hematological malignancies.

Tumor cells may express ligands (PD-L1, PD-L2, B7-H3) belonging to the immune checkpoint family, i.e. inhibitory pathways that limit the duration and amplitude of anti-tumor responses.

B7-H3 not only weakens the strengthening of the NK- (and T-) mediated anti-tumor responses, but also exerts a direct pro-tumoral activity.

Tumor cells can constitutively express inhibitory ligands or increase their expression in response to immunostimulatory cytokines (IFN-γ, TNF-α) that are released by activated NK (and T) cells, a phenomenon called "tumor adaptive immune resistance".

IFN-γ, besides inducing PD-Ls expression, is also a potent inducer of HLA- II that can be recognized by LAG-3, an additional inhibitory mechanism that recently emerged together with TIGIT/PVR and TIM-3/galectin-9 interactions.

Future directions

When designing immunotherapies, we can't neglect the complexity of the immune-suppressive milieu characterizing the tumor microenvironment. Thus, NK cell-based therapeutic protocols should combine optimal activation strategies, the selection of the best NK cell subpopulation, consider the *in vivo* persistence of NK cells, and their chemokine receptor repertoire. Valuable adjuvants may include the use of humanized mAb to disrupt immune checkpoints pathways and/or to hinder the effects of soluble factors that *in vivo* may dampen the NK cell activity against tumors.

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Conflict Of Interest

A.M. is a founder and shareholder of Innate-Pharma (Marseille, France). The remaining authors declare no conflicts of interest.

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Review

Main NK cell receptors and their ligands: regulation by microRNAs

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Abstract: The NK cells functions are finely tuned by several kinds of inhibitory and activating receptors, whose pattern of expression characterizes different NK subpopulations and varies with the cell activation status. MicroRNAs have an important role in tightly regulating the expression of NK receptors and, analogously, the expression of their ligands in target cells. The relevance of the microRNA-mediated control is highlighted by the dysregulation of these pathways observed in cancer and virus-infected cells. Here we review our current knowledge of the microRNAs involved in the regulation of NK receptors, as well as that of the corresponding cellular ligands.

Keywords: NK cells; microRNAs; NK receptors; NK receptor ligands; TGF-β1

1. Introduction

Natural Killer (NK) cells are innate lymphoid cells endowed with several effector and regulatory functions, including cytotoxic activity against virus infected and tumor-transformed cells. Although there is a growing knowledge of the phenotypic heterogeneity of the NK cell population [1], two major subsets of circulating NK cells have been described, $CD56^{bright}$ $CD16^{low/neg}$ and $CD56^{dim}$ $CD16^{pos}$ cells, which have different function and tissue distribution. $CD56^{bright}$ NK cells, which release large amount of soluble factors in response to pro-inflammatory cytokines, are poorly represented in peripheral blood, while largely populating secondary lymphoid organs. Conversely, $CD56^{dim}$ cells, which are highly cytotoxic effectors, represent the largest percentage of circulating

NK cells.

The NK cell functions are finely regulated by a variety of germline-encoded inhibitory and activating receptors and by different cytokines, produced in the early or in the late phase of immune responses. All these cytokines other than potentiate NK cell responsiveness towards transformed cellular targets, stimulate the production of chemokines and cytokines (IFN-γ, TNF-α) crucial for shaping both innate and adaptive immunity [1,2].

The main inhibitory NK receptors include: Killer-cell immunoglobulin-like receptors (KIRs), CD94/NKG2A and LILRB1, MHC class I-specific receptors that physiologically maintain self-tolerance; PD-1, TIM-3 and LAG-3 that belong to the immune checkpoint family, inhibitory pathways that are induced/up-regulated during immune responses and limit the duration and amplitude of the immune responses, thus minimizing tissue damage. Activating signals are mediated by a plethora of activating receptors and co-receptor including NKp46, NKp30 and NKp44 (collectively termed Natural Cytotoxicity Receptors, NCR), NKG2D and DNAM-1. The activating receptors recognize non-MHC ligands up-regulated or *de novo* expressed by cells upon cellular stress, tumor transformation or viral infection. The modality of NK cell activation, well depicted by the rheostat model [3,4], depends on the cytokine milieu as well as on the presence/absence and surface densities of the different ligands on neighboring healthy or altered cells.

Different mechanisms regulate the expression of receptors, on effector NK cells, or ligands on potential targets. Among these, microRNA-mediated mechanisms that regulate gene expression at the post-transcriptional level. MicroRNA (miRNAs) are small RNAs, processed from stem-loop regions of longer RNA transcripts, which can interact with the 3'-Untranslated Region (3'-UTR) of target mRNAs via sequence complementarity, thus down-regulating gene expression through translation repression and/or mRNA degradation [5]. The importance of this kind of regulation is underlined by the fact that tumors and viruses evolved mechanisms to negatively affect NK activity acting through these pathways.

This review synthetically describes the current knowledge of miRNAs targeting molecules fundamental for the NK cell effector functions and trafficking.

2. NK cell receptors and miRNAs

Several miRNAs have been described that target different NK receptor pathways. These include activating receptors (NKG2D), the DAP12 signaling molecule, inhibitory receptors (KLRG1), immune checkpoint receptors (TIM-3, PD-1 and CTLA-4), chemokine and cytokine receptors (CX_3CR1, IL-2Rγ). Interestingly, TGF-β1, an immune-modulatory cytokine highly produced and activated in the tumor microenvironment [6], has been shown to hamper NK cells activity down-regulating different receptors via distinct miRNA pathways (Figure 1).

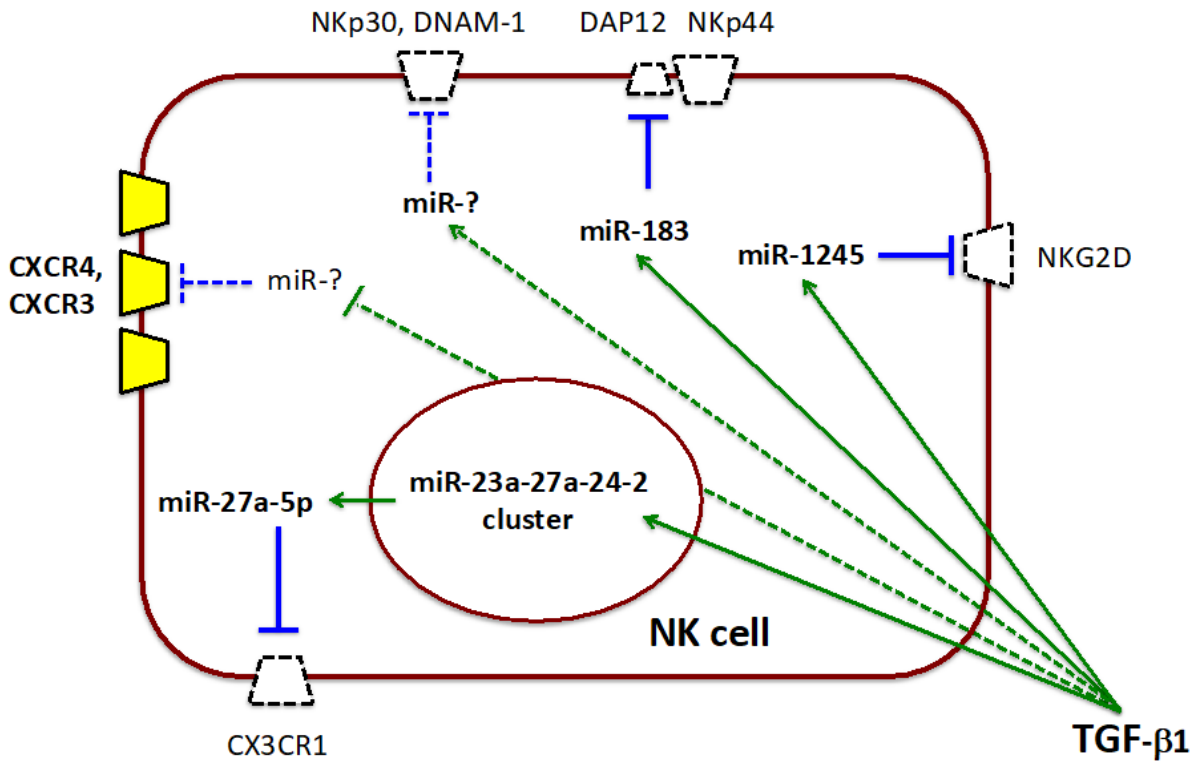

Figure 1. TGF-β1 modifies the receptors expression in human NK cells. The TGF-β1 contained in the tumor microenvironment reduces the expression of NKG2D, DAP12/NKp44 and CX_3CR1 in NK cells via different miRNA-mediated pathways. The involvement of miRNAs in the TGF-β1-mediated up-regulation of CXCR4 and CXCR3 [9] or down-regulation of NKp30 [8] and DNAM-1 [91] remains to be determined.

2.1. NKG2D

NKG2D (CD314) is a C-type lectin-like receptor expressed on NK and $CD8^+$ T cells. It is an activating receptor able to detect and promote the elimination of damaged, transformed, and pathogen-infected cells by recognizing stress-inducible ligands belonging to the MIC (MICA and MICB) and ULBP (ULBP1–6) families in humans [7]. *In vitro* studies showed that the surface expression of NKp30 and NKG2D is significantly down-regulated by conditioning NK cells with TGF-β1 or TGF-β2 [8,9]. Moreover, subsequent studies highlighted the presence *in vivo* of $NKG2D^{low}$ NK cells in cancer patients, ascribable to TGF-βactivity. One mechanism responsible for the TGF-β-mediated NKG2D down-regulation has been associated to the impairment of transcriptional and translational level of DAP10, the signaling subunit associated with human NKG2D [10]. Another mechanism has been showed by Espinoza et al. [11] who reported that TGF-β1 causes an increase of miR-1245 in NK cells. Overexpression of miR-1245 induced a down-regulation of NKG2D at the cell surface level, but had no effect on other activating receptors such as NKp30, NKp44 and NKp46. Moreover, NK cells overexpressing miR-1245 had lower cytotoxicity against target cells expressing NKG2D ligands. MiR-1245 was shown to directly target and down-regulate the NKG2D mRNA.

2.2. DAP12

DAP12 (DNAX activating protein of 12 kDa) (also termed killer cell activating receptor-associated protein, KARAP) is a transmembrane protein containing an immune tyrosine-based activation motif (ITAM) in its cytoplasmic domain [12]. DAP12 is a fundamental signaling molecule that mediates a broad array of biological functions by associating with different activating receptors expressed in cells of lymphoid and myeloid lineage [12]. These receptors include the activating isoform of KIRs, CD94/NKG2C, and NKp44, the latter being expressed by NK cells upon activation. Donatelli et al. [13] reported that TGF-β1 down-regulates the DAP12 level in NK cells. MiR-183, which was up-regulated by TGF-β1, was shown to directly target DAP12 mRNA. NK cells overexpressing miR-183 had reduced DAP12 (both mRNA and protein) as well as decreased NKp44 surface levels. NK92 cells transduced with miR-183 were less efficient in killing Raji cells (which do not express NKG2D ligands) with respect to control lentiviral transduced cells. Interestingly, NK cells infiltrating lung cancers were shown to have a diminished DAP12 expression.

2.3. KLRG1

KLRG1 is a C-type lectin-like inhibitory receptor, containing an immune tyrosine-based inhibitory motif (ITIM), which binds to members of the cadherin family leading to inhibition of NK and T cell function. It is predominantly expressed by NK cells with a mature phenotype [14] and it is strongly induced by viral and other infections [15]. Cipolla et al. [16] reported that miR-584-5p targets KLRG1 and that overexpression of miR-584-5p in peripheral blood mononuclear cells (PBMC) causes a decrease of KLRG1 mRNA expression. Interestingly, a 3'-UTR KLRG1 polymorphism, rs1805672, associated to the autoimmune disease Pemphigus foliaceus, abolishes the binding of the miRNA, thus causing the loss of the miRNA-mediated control of KLRG1 expression. Authors suggested a link between the loss of the miRNA-target interaction and predisposition to the autoimmune disease.

2.4. TIM-3

TIM-3 belongs to the T cell immunoglobulin and mucin domain (TIM) family. It is expressed on the surface of several immune cells, including NK cells. Upon activation by the C-type lectin galectin-9 ligand, TIM-3 functions as an inhibitory receptor on NK cells by reducing their cytotoxicity and cytokine production [17]. Cheng et al. [18] reported that TIM-3 and T-bet expression in NK cells from chronically HCV-infected patients is up-regulated compared to NK from healthy controls, while miR-155 is down-regulated. The opposite regulation of TIM-3/T-bet and miR-155 was recapitulated *in vitro* by incubating primary NK cells or the NK92 cell line with Huh-7 hepatocytes expressing HCV. MiR-155 transfected in NK92 cells reduced TIM-3 and T-bet and enhanced IFN-γ expression. Authors suggest that TIM-3 expression is indirectly regulated by miR-155.

2.5. PD-1

PD-1 is an immune checkpoint receptor evolutionary related to the CD28 family. It recognizes

two cellular ligands, PD-L1 and PD-L2, and can be expressed on several cell types including T, B, NK, NKT and myeloid cells. Regarding NK cells, its expression seems to be confined to terminally differentiated $NKG2A^{neg}$ KIR^{pos} $CD57^{pos}$ NK cells, whose antitumor activity can be partially restored *in vitro* by antibodies disrupting the PD-1/PD-Ls interactions [19]. Timing in the expression of PD-1 is in line with the PD-1-dependent regulatory activity, which occurs in the later phases of the immune response [20]. To date, no miRNAs modulating PD-1 have been described in NK cells. Nevertheless, miR-4717, regulating only PD-1 variants containing a specific polymorphism in the 3'-UTR, has been described in total lymphocytes [21]. Moreover, miR-138 has been reported to modulate CTLA-4 and PD-1 expression in mouse and human $CD4^+$ T cells [22], and miR-28 has been described to regulate PD-1 in mouse $CD4^+$ T cells [23].

2.6. CTLA-4

CTLA-4 (CD152) is an immune checkpoint receptor binding the CD80 and CD86 ligands with higher affinity than CD28. It is expressed in Treg and activated T cells, while its expression in NK cells has been detected in mouse, but not in human [24]. CTLA-4 is targeted by miR-155, and in stimulated naive T_H cells transfection with the miR-155 precursor down-regulates CTLA-4 expression [25]. Moreover, as for PD-1, CTLA-4 is regulated by miR-138 in mouse and human $CD4^+$ T cells [22].

2.7. CX_3CR1

CX_3CR1 is a chemokine receptor expressed by different immune cell types, including monocytes, dendritic cells (DCs), T, and NK lymphocytes, which binds CX_3CL1 (also known as fractalkine). CX_3CR1, with other chemokine receptors, drives, at steady state, NK cell localization in peripheral tissues, and promotes their migration under inflammatory conditions [26]. Moreover, it has been shown that CX_3CR1, CXCR4 and S1P5 regulate NK cells homing and migration from the bone marrow (BM) [27,28]. It has been demonstrated that the surface expression of CX_3CR1 was down-regulated in NK cells co-cultured with SH-SY5Y neuroblastoma (NB) cells under trans-well conditions. Notably, CX_3CR1 expression was reduced in NK cells from NB patients compared with healthy controls [9]. TGF-β1 released by NB cells has been shown to be responsible for the observed CX_3CR1 down-regulation, as also confirmed *in vitro* using recombinant TGF-β1. Looking for the mechanisms involved, TGF-β1 was found to determine a significant increase of miR-27a-5p, which targeted the CX_3CR1 mRNA in NK cells [29]. Up-regulation of miR-27a-5p was due to the TGF-β1-dependent up-regulation of miR-23a-27a-24-2 cluster, which encodes, among others, miR-27a-5p. As expected, inhibition of miR-27a-5p expression in NK cells caused an up-regulation of the CX_3CR1 mRNA. Conversely, a reduction of CX_3CR1 surface expression was observed in CX_3CR1-expressing HEK293T cells following their treatment with miR-27a-5p mimic. In agreement with the original findings [9], NK cells cultured in the presence of SH-SY5Y NB cells exhibited a significant up-regulation of miR-27a-5p and down-regulation of CX_3CR1 mRNA [29].

2.8. IL-2Rγ

IL-2Rγ is a transmembrane protein belonging to the cytokine receptor gene superfamily. It is the

γ component of multiple cytokine receptors, including those for IL-2, -4, -7, -9, -15, and -21, which are expressed on the surface of lymphocytes and other hematopoietic cells. Lack of IL2Rγ due to mutations of the encoding gene is responsible for X-linked severe combined immunodeficiency (X-SCID), a cellular and humoral immunodeficiency with a near-complete absence of NK and T cells and nonfunctional B cells [30]. Yun et al. [31] reported that miR-583 targets IL-2Rγ. Differentiating NK cells transfected with miR-583 mimics showed a decreased surface expression of IL-2Rγ and defective differentiation. In particular, the miR-583 overexpressing NK cells showed decreased NKG2D, NKp30 and NKp46 expression and reduced capacity to kill target cells and to produce IFN-γ [31].

3. NK receptor ligands and miRNAs

A number of miRNAs have been identified as regulators of ligands of NK cell receptors. Targeted molecules include ligands for MHC class I-specific (HLA-G, HLA-C, HLA-E) and non-specific (MICA, MICB, ULBPs) receptors, as well as the immune checkpoint ligands PD-L1, B7-H3, CD80 and CD86. In cancer cells modification of miRNA expression may represent a common mechanism of escape from the NK-mediated immune surveillance (Figure 2).

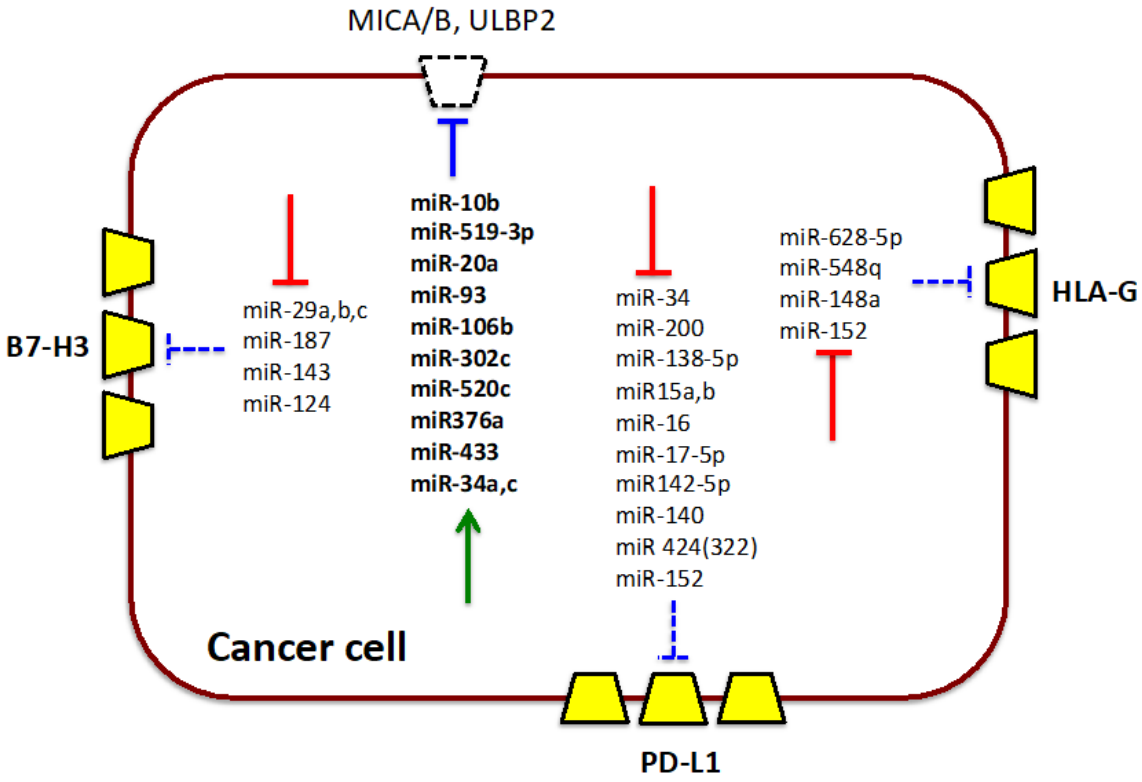

Figure 2. Altered ligands expression in tumor cells. Cancer cells can hamper NK cytotoxicity by up- or down-regulating a number of miRNAs, which reduce the expression of ligands of activating receptors or increase that of inhibitory ones, respectively. The latter includes the immune checkpoint ligands PD-L1 and B7-H3.

3.1. NKG2D ligands

NKG2D ligands MICA, MICB, and ULBPs can be regulated by several miRNAs, of cellular or viral origin [32] that are potentially able to protect cancer cells and virus infected cells from NK cell cytotoxicity [7,32,33]. Stern-Ginossar et al. [34] reported 7 miRNAs regulating MICA and MICB expression (miRNAs miR-17-5p, miR-20a-5p, miR-93-5p, miR106b-5p, miR-372, miR373-3p, miR-520d-3p). Overexpression of these miRNAs *in vitro* and *in vivo* resulted in down-regulation of MICA and MICB expression and in lower susceptibility to NKG2D-dependent killing by human NK cells. On the other hand, three different viral miRNAs (miR-UL112, miR-BART2.5p and miR-K12-7), all derived from herpesviridae, targeted MICB, while a miRNA from polyoma viruses JCV and BKV targeted ULBP3 [32,33]. Two miRNAs (miR-376a and miR-433) were shown to regulate MICB and to decrease NK-mediated cytotoxicity against colon carcinoma cells [35]. MiR-34a and miR-34c were reported to target ULBP2 and, when overexpressed in a melanoma cell line, down-regulated its expression at the protein level. Moreover, miR-34c transfected melanoma cells displayed reduced susceptibility to NK cells cytotoxicity [36]. Other miRNAs (miR-10b, miR-519-3p, miR-20a, miR-93, miR-106b, miR302c, miR-520c), which were found overexpressed in various tumors, have been shown to down-regulate MICA, MICB and ULBP2, and, consequently, to reduce the NK cell-mediated killing of target cells [37–42].

3.2. HLA-G

HLA-G is a non-classical HLA class Ib molecule, expressed mainly in the placental trophoblasts, where is thought to provide immune protection to the embryo from the decidual NK cells, the major lymphocyte population at the fetal-maternal interface. HLA-G is also expressed by tumors and virus-infected cells [43]. HLA-G is recognized by LILRB1 and KIR2DL4 inhibitory receptors on human NK cells [44] and its soluble form has been described to regulate the chemokine receptor repertoire [45]. Several miRNAs down-regulate HLA-G expression: miR-148a, miR-152 (belonging to the same miRNA family), miR-133a, miR-548q, miR-628-5p and miR-365 [44,46–48]. An inverse correlation was reported between miR-628-5p and HLA-G in primary renal cell carcinoma tumors and cell lines [49]. In the case of miR-148a, miR-152 and miR-548q, it has been experimentally shown that they induce in target cells HLA-G down-regulation and increased susceptibility to the cytolytic activity of $LILRB1^{+}$ NK cells [44,47,49]. The long noncoding RNA HOTAIR has been shown to bind miR-148a in cervical cancer [50] and miR-152 in gastric cancer cells [51], thus up-regulating HLA-G. TGF-β1 has been reported to cause a down-regulation of miR-152 inducing an up-regulation of HLA-G in gastric cancer cell lines [52].

3.3. HLA-C

HLA-C are classical HLA class I molecules recognized by inhibitory KIRs. In particular, KIR2DL1 binds C2 allotypes characterized by a lysine in position 80, whereas KIR2DL2 and KIR2DL3 recognize C1 allotypes sharing an asparagine in position 80 [53]. Interestingly, miR-148a is able to bind not only HLA-G, but also HLA-C. Kulkarni et al. [54] reported that a polymophism in the HLA-C 3'-UTR regulates binding capability of miR-148a to its target site, resulting in relatively low HLA-C surface expression of alleles that bind this miRNA and high expression of HLA-C

alleles that escape post-transcriptional regulation.

3.4. HLA-E

The non-classical HLA-E binds a restricted subset of peptides encoded by the leader sequence of other class I molecules. It is recognized by the inhibitory CD94/NKG2A and activating CD94/NKG2C heterodimeric receptors, both expressed by T cells and NK cells [55]. The human cytomegalovirus [HCMV] encodes UL40, a peptide stabilizing HLA-E and driving the proliferation of CD94/NKG2C$^+$ NK cells [56]. Interestingly, Nachmani et al. [57] showed that the short form of the RNA-editing enzyme ADAR1 induced editing of miR-376a in HCMV$^+$ cells. Edited miR-376a down-regulated HLA-E expression rendering HCMV infected cells susceptible to elimination by NK CD94/NKG2A$^+$ NK cells.

3.5. PD-L1

PD-L1 and PD-L2 are the two ligands of the PD-1 inhibitory receptor, which is expressed by activated NK and T cells [58,59]. Under physiological conditions, the PD-1/PD-Ls interactions control the duration and amplitude of immune responses, preventing autoimmune reactions. In cancer cells the expression of PD-Ls plays a crucial role in immune evasion. Several miRNAs regulating PD-L1 have been described, suggesting a tight control of its expression. In cancer cells PD-L1 expression is accompanied by miRNAs down-regulation [60–62]. MiR-513 has been the first miRNA reported to regulate PD-L1 and its inhibition in cholangiocytes induced PD-L1 protein expression [63]. In acute myeloid leukemia samples an inverse correlation was reported between miR-34 and PD-L1 expression and miR-34 was found to regulate PD-L1 expression in leukemia cell lines [64]. An inverse correlation was also reported between miR-200 and PD-L1 expression in lung adenocarcinoma samples. MiR-200 suppressed PD-L1 expression in murine and human mesenchymal lung cancer cell lines, while its down-regulation increased PD-L1 expression causing CD8$^+$ T cells immune suppression [65]. MiR-138-5p was also found to target PD-L1. An inverse correlation was reported between miR-138-5p and PD-L1 expression in human colorectal cancer (CRC) samples and miR-138-5p overexpression suppressed CRC cell tumorigenicity [66]. Other miRNAs have been described as regulators of PD-L1 in various cancer cells, miR-15a, miR-15b and miR-16 in malignant pleural mesothelioma cell lines [67], miR-17-5p in metastatic melanoma [68], miR-142-5p in a pancreatic cancer cell line [69], miR-140 in non-small cell lung cancer (NSCLC) cell lines [70]. MiR-424(322) regulated PD-L1 and CD80 in ovarian cancer and high levels of this miRNA correlated with increased progression-free survival of patients [71]. Expression of miR-152 was low in gastric carcinoma compared to controls, and miR-152 mimics inhibited PD-L1 expression in gastric cancer cell lines with increased T cell proliferation and function [72]. MiR-324-5p and miR-338-5p targeted PD-L1 and dendritic cells expressing these miRNAs displayed reduced surface expression of PD-L1 [73].

3.6. B7-H3

B7-H3, a member of the B7 superfamily [74,75], is the orphan ligand of an inhibitory receptor expressed by human NK and CD8$^+$ T cells [74,76]. B7-H3 is considered as a new member of the

immune checkpoint family [77]. Indeed, it has been shown to inhibit cytotoxicity and protect neuroblastoma (NB) (and other tumor cells) from NK cell-mediated lysis [74,76]. B7-H3 also promotes tumor invasiveness [78]. In line with these pro-tumoral properties, high B7-H3 expression correlates with poor prognosis in several tumor histotypes [78,79]. B7-H3 expression is regulated by several miRNAs. Xu et al. [80] reported that miR-29a, b, c are down-regulated in several B7-H3 expressing tumor tissues compared to normal tissues. MiR-29a targets B7-H3 in neuroblastoma cell lines [80] and down-regulation of miR-29a in central nervous system NB metastasis is associated with up-regulation of different molecules, including B7-H3 [81]. MiR-29c expression was inversely correlated to B7-H3 expression in melanoma [82] and was reported to target B7-H3 in breast cancer, where miR-29c expression correlated with increased survival [83]. Another miRNA targeting B7-H3 is represented by miR-187. MiR-187 was down-regulated in clear cell renal cell carcinoma [ccRCC] compared to normal tissues. Overexpression of miR-187 (as well as silencing of B7-H3) decreased cell migration in ccRCC cell lines [84]. Interestingly, in CRC cell lines, miR-143 was down-regulated by TGF-β1, thus causing the up-regulation of both its targets B7-H3 and B7-H4 and leading to tumor immune evasion [85]. MiR-124 targeted B7-H3 in osteosarcoma [OS] cells and inhibited their growth and invasive ability. Moreover, miR-124 was down-regulated in OS clinical specimens, with an inverse correlation between miR-124 and B7-H3 protein expression [86].

3.7. CD80 and CD86

The expression of CD80 and CD86, CTLA-4 ligands, can be modulated by miRNAs. Besides miR-424(322) that regulates both PD-L1 and CD80 (see above) [71], miR-134 has been shown to bind CD86 mRNA suppressing its expression. Transfection of miR-134 in a melanoma cell line caused reduction of CD86 at the mRNA and protein level [87].

4. Conclusion

What clearly emerges from the recent literature on the regulation of NK receptors and ligands expression is a central role of miRNAs in the maintenance of a delicate equilibrium that can be heavily perturbed by tumor transformation or viral infection.

To escape NK cell immunosurveillance, tumor and virus infected cells evolved mechanisms to deregulate the expression of genes coding for molecules important for effector/target interactions [88]. These mechanisms often use the miRNA machinery. Pathological cells can affect NK-mediated recognition by up- or down-regulating miRNA targeting activating or inhibitory receptor ligands, respectively. Tumor cells can also act on NK cells by producing immunomodulatory factors, such as TGF-β1 that, influencing the expression of different receptors via distinct miRNA-pathways, realizes a combined inhibitory action. Other mechanisms can add levels of complexity to the action of the miRNA machinery, as the long noncoding RNAs sequestering miRNAs or the polymorphisms in the 3'-UTR of miRNA target genes.

The link between miRNA derangement and unpaired NK immunosurveillance makes miRNAs potential therapeutic targets [89]. MiRNA mimics or antimiRs can be used to increase or decrease the activity of miRNAs with tumor suppressive or oncogenic function. However, susceptibility to degradation in biological fluids and poor delivery to target sites might severely hamper the use of miRNAs in therapy. Chemical modifications of the miRNA backbone and the use of specific vehicles,

as nanoparticles or minicells, have been developed to confer stability to the miRNAs. Moreover, specific molecules, such as antibodies, can be conjugated to the miRNAs or to the vehicles to ensure an efficient and specific delivery. Avoiding toxicities and off-target effects are additional challenges for a miRNA-based therapeutic approach.

In spite of these difficulties, two different phase I clinical trials treating cancer patients have been performed using miRNAs as therapeutic agents. The former study (NCT01829971) used miR-34 mimics encapsulated in lipid nanoparticles, without the association with specific targeting molecules, to treat patients affected by several solid tumors and hematological malignancies. Unfortunately, it was terminated due to immune related serious adverse events. In the latter study (NCT02369198), miR-16 mimics encapsulated in minicells targeted to EGFR-positive cancer cells by an anti-EGFR antibody have been used to treat patients with malignant pleural mesothelioma and non-small cell lung cancer. The study was regularly completed, establishing a dose of miRNA mimic which was well tolerated and accompanied by early signs of antitumor activity [90].

Therefore, combination of targeted miRNA-based therapies with other therapeutic interventions represents a possible strategy to reduce tumor aggressiveness and to potentiate NK cell-mediated responses against cancer.

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Conflict of interest

All authors declare that they have no conflict of interest in this paper.

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REVIEW
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Molecular Mechanisms Directing Migration and Retention of Natural Killer Cells in Human Tissues

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A large body of data shows that Natural Killer (NK) cells are immune effectors exerting a potent cytolytic activity against tumors and virus infected cells. The discovery and characterization of several inhibitory and activating receptors unveiled most of the mechanisms allowing NK cells to spare healthy cells while selectively attacking abnormal tissues. Nevertheless, the mechanisms ruling NK cell subset recirculation among the different compartments of human body have only lately started to be investigated. This is particularly true for pathological settings such as tumors or infected tissues but also for para-physiological condition like pregnant human uterine mucosa. It is becoming evident that the microenvironment associated to a particular clinical condition can deeply influence the migratory capabilities of NK cells. In this review we describe the main mechanisms and stimuli known to regulate the expression of chemokine receptors and other molecules involved in NK cell homing to either normal or pathological/inflamed tissues, including tumors or organs such as lung and liver. We will also discuss the role played by the chemokine/chemokine receptor axes in the orchestration of physiological events such as NK cell differentiation, lymphoid organ retention/egress and recruitment to decidua during pregnancy.

Keywords: natural killer cells, chemokines and chemokine receptors, migration and residency, tumor and inflammation, pregnancy

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INTRODUCTION

The initial view describing Natural Killer (NK) cells as a quite homogeneous $CD3^{neg}$ $CD56^{+}$ circulating lymphocyte population has been largely overcome. NK cells have been recently included in a wider innate lymphoid cell (ILC) family, and circulating cells are just the tip of an iceberg formed by a conspicuous and heterogeneous lymphoid population colonizing both, lymphoid and non lymphoid tissues (1–3). Moreover, cytometry by time-of-flight (CyTOF) highlighted the existence in peripheral blood (PB) of a single individual of at least 30,000 different NK cell phenotypes (4). These findings consolidate the concept that observed phenotypic and functional NK cell status actually represents a single crystalized picture of a very dynamic process. Nevertheless, in healthy individuals, two main circulating PB NK cell populations have been extensively studied, $CD56^{bright}$ and $CD56^{dim}$ NK cells, which represent sequential stages of maturation and show a dichotomy in phenotypic and functional properties (5). These include the

expression of MHC class I-specific inhibitory Killer Ig-like Receptors (KIRs), restricted to $CD56^{dim}$ NK cells that represent the majority of cells circulating in blood. KIRs are involved in NK cell "education," a phenomenon that provides the basis of self-tolerance and generates "armed" cells, i.e., NK cells fully responsive to the engagement of activating receptors (i.e., NCR, NKG2D, and DNAM-1) (6, 7). $CD56^{dim}$ NK cells also express high levels of CD16, thus exerting strong antibody-dependent cellular cytotoxicity (ADCC). Moreover, they efficiently respond to cytokines stimulation and are characterized by a chemokine receptor repertoire giving them the potential to colonize lymphoid and non-lymphoid tissues in response to a proper chemokine milieu.

The composition of the milieu can greatly vary in perturbed tissues. This justifies the prevalence in some tumors of immature, poor cytolytic $CD56^{bright}$ NK cells that are undetectable in matched healthy tissues (8). Tumor parenchyma, as well as the immune cells participating to the inflammatory processes, may change the microenvironment providing NK cells with a plethora of stimuli. These include membrane-bound or soluble molecules such as chemokines or cytokines (TGF-β, IL-12, IL-18), which either promote or dampen innate and adaptive immune responses. Cytokines, in addition to shape the functional activity of NK cells, modify their chemokine receptor repertoire altering their native migratory potential (9–13) and at the same time provide signals essential to generate, expand and recall memory NK cell populations (14). Interestingly, recent data showed that non-hematopoietic organs such as liver can be colonized by peculiar tissue resident NK cell populations that belong to the memory NK cell reservoir able to mediate "recall" responses (15).

Here, we will recapitulate studies that analyzed the main mechanisms regulating NK cell trafficking in lymphoid and non-lymphoid tissue under either steady state or "perturbed" conditions, including tumors, inflammation and pregnancy.

DEFINING DYNAMICS OF NK CELLS IN HEALTHY TISSUES

NK cells are not exclusively found in PB but populate different tissues and organs. The traditional view of NK cells as "armed" effector cells, which patrol human body through blood ready to extravasate to the site of injury, has been partially revisited and a growing number of studies show that NK cells might also stably reside in most peripheral tissues, under steady-state conditions.

Until recently, the task of depicting NK cell distribution in human compartments has suffered from several methodological shortcomings. Earlier analyses often relied on the use for NK cells detection of markers poorly specific and/or unable to distinguish the two main NK cell subsets, i.e., $CD56^{bright}$ $CD16^{low/neg}$ $Perf^{low}$ and $CD56^{dim}$ $CD16^{pos}$ $Perf^{high}$. The advent of new OMICS technologies, and the possibility to perform single-cell analyses have expanded our understanding on the distribution of NK cells across human body. Indeed, in the recent years, our knowledge about NK cell diversity has further increased with the identification of NK cell subsets specifically populating various peripheral solid organs, such as lung, liver, lymphoid tissues, and uterus. These findings have challenged the classical view of NK cells as a lineage comprising a relatively homogeneous population of cells with similar functions and longevity. Nonetheless, at variance with B and T cells, we know little about recirculation and trafficking of NK cells across peripheral tissues. Although NK cells express an ample array of chemotactic receptors, the role of the different chemokines in guiding *in vivo* the distribution of NK cells through the body compartments still remains unclear. The distribution of NK cells seems to be subset-specific in mouse, as different NK cell subsets showed organ-specific localizations (16). Conversely, this issue has been poorly investigated in the human system. As the two major PB-NK cell subsets display a chemokine receptors pattern that only partially overlaps, they may have a peculiar tissue-specific compartmentalization (**Figure 1**). PB-$CD56^{bright}$ NK cells are uniquely characterized by the expression of CCR7, CXCR3, and L-selectin (CD62L), which justify their abundance in secondary lymphoid tissues (SLTs). Conversely, PB-$CD56^{dim}$ NK cells, despite sharing the CXCR4 receptor with $CD56^{bright}$ NK cells, are equipped with receptors specific for inflammatory chemokines, such as CXCR1, CXCR2, CX_3CR1 (8, 16, 17). Additionally, $CD56^{dim}$ NK cells can migrate in response to factors that do not belong to the chemokine superfamily. These include the proinflammatory protein chemerin and the sphingosine 1-phosphate (S1P) molecule that affect trafficking of NK cells during inflammation or steady-state conditions, respectively (18, 19). Based on the different expression of chemotactic receptors, the tissue distribution of human NK cell subsets observed under steady-state conditions is dependent on the expression of local tissue-specific environmental signals. In order to shed light on the mechanisms lying behind the migratory properties of PB NK cells, a wide array of samples derived from different body compartments was analyzed to investigate the presence and distribution of functionally different NK cell subsets (8). The study showed that the relative distribution of $CD56^{bright}$ and $CD56^{dim}$ NK subsets in the various human districts does not parallel that in PB. $CD56^{dim}$ NK cells represent the major NK cell subset in bone marrow (BM), lung, spleen, subcutaneous adipose tissue and breast tissue, whereas $CD56^{bright}$ NK cells abundantly outnumber cytotoxic NK cells in gastric and intestinal mucosa associated lymphoid tissues (MALTs), liver, uterus, visceral adipose tissue, adrenal gland, and kidney (8, 20, 21). Importantly, the relative distribution of the two main NK cell subsets matched with the specific patterns of chemotactic factors expressed in the tissues (8).

A main question arising from the detection of NK cells in many organs is whether NK cells stably reside in those tissues or could eventually exit and recirculate. Studying the dynamics of NK cells under steady-state conditions is limited by the difficulty of having access to samples from human body districts. On this regard, useful hints may be derived from studies in which human subjects have been treated with monoclonal antibodies directed against molecules pivotal in lymphocytes migration, such as integrins. This is the case of natalizumab, a humanized monoclonal antibody directed against the α4-chain of VLA-4 (α4β1) and α4β7 integrins, widely expressed on many different lymphocyte populations including T cells,

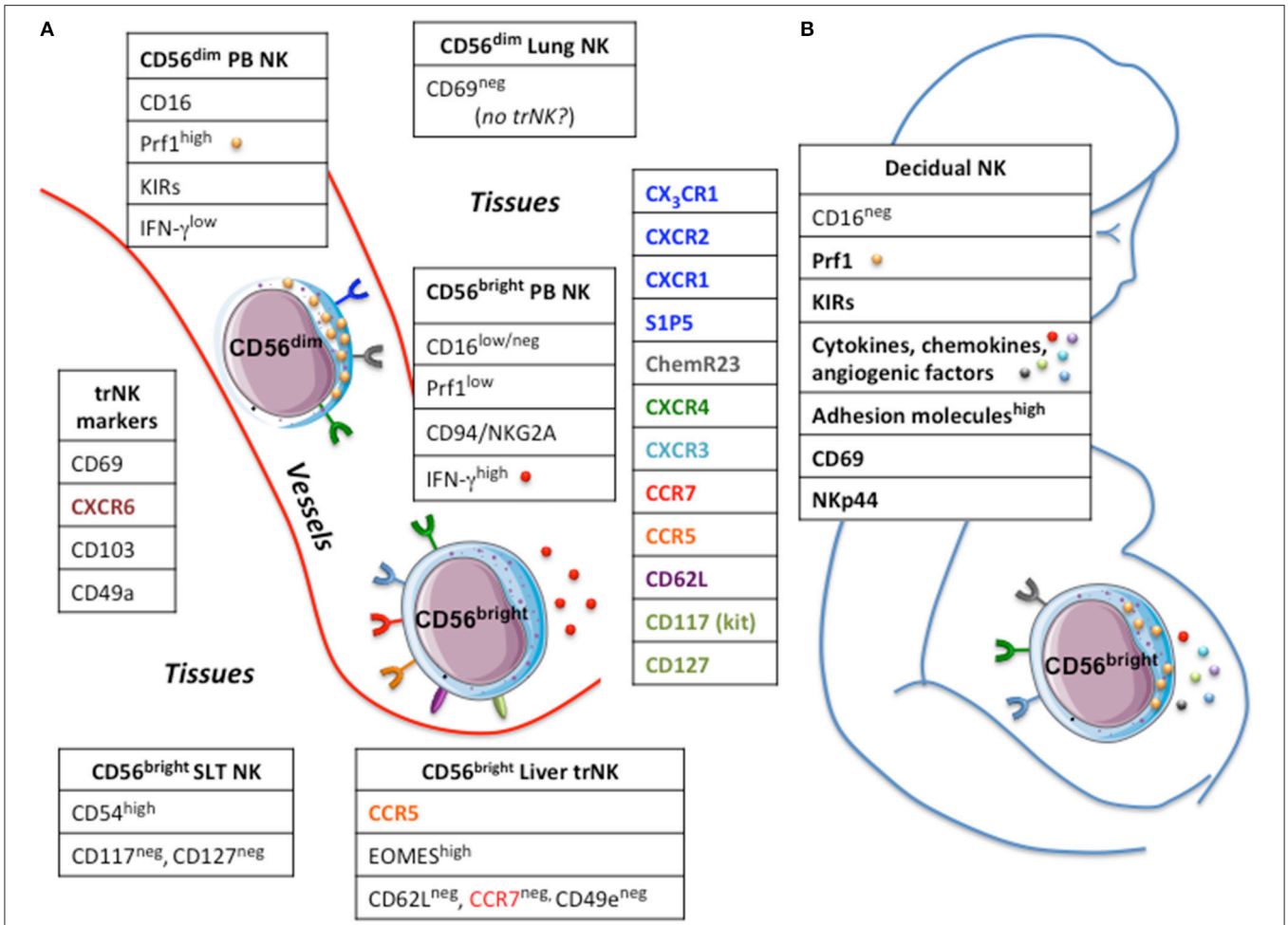

FIGURE 1 | Circulating and tissues resident NK cells. **(A)** In human two main NK cell subsets, CD56bright and CD56dim, can be detected in peripheral blood (PB NK) having a different repertoire of chemokine receptors. Tissue resident (tr) NK cells share the expression of certain markers but express molecules typical of the hosting tissue.**(B)** large numbers of NK cells populate the decidua, particularly in the first trimester of pregnancy. Decidual NK cells have unique phenotypic and functional characteristics, which contribute to support nutrition of the fetus, ensure maternal-fetal tolerance and control viral infections. Prf1, perforin; KIRs, killer cell immunoglobulin-like receptors; SLT, secondary lymphoid tissues.

B cells, and NK cells as well as on a majority of monocytes and macrophages. Interestingly, it has been reported that 1-year treatment with natalizumab in multiple sclerosis patients resulted in a pronounced accumulation (almost 2-fold increase compared to baseline levels) of NK cells in PB (22), which then gradually decreased upon treatment interruption (23). These data are in evident agreement with a dynamic passage of circulating NK cells across the endothelial barriers for patrolling peripheral tissues, although it remains to be determined whether it might occur also in steady-state or just under inflammatory conditions.

In addition to extravasation from PB to solid tissues, NK cells may eventually egress from peripheral tissues and trafficking to SLT. This re-circulation has been suggested by the direct investigation of afferent lymph draining from normal skin (24) and analysis of cellular content in seroma fluid upon axillary lymph nodes (LN) dissection, which represents an accumulation of *bona fide* afferent lymph (25, 26). Interestingly, most seroma NK cells expressed high level of CCR7 and CD62L, as well as CXCR4, CXCR3, a chemokine receptor repertoire identifying lymphocyte populations migrating toward SLTs. These data indicate that high endothelial venules (HEVs) might not represent the only route for NK cell entrance in SLTs. Conversely, very little information is available regarding the egress of NK cells from SLTs. It has been described in the murine model that changes in responsiveness of sphingosine-1 phosphate receptor 5 (S1P5) to its ligand (S1P) play a key role in allowing NK cell egress via lymphatics (27). However, whether this mechanism might also be effective in human has not yet been confirmed. Notably, NK cells have been detected in efferent lymph fluid and NK cells exiting from LN have a phenotype slightly different from that of NK cells found within SLTs. In particular, a portion of NK cells express significant amounts of KIR and CD16, implying that $CD56^{bright}$ NK cells might acquire

these molecules in the LN during inflammation and then egress through the efferent lymph for recirculating in PB (28).

All these previous studies have so far depicted the distribution of the two main "conventional" human NK cell subsets across the human body (8, 29). Recently, this issue reached a higher level of complexity because of data showing that various body districts harbor "unconventional" subsets of NK cells that apparently do not recirculate in the blood or lymphatics and adopt a unique phenotype that is distinct from that of circulating NK cells. Tissue residency has been described for NK cells as well as for other "helper" innate lymphoid cells (ILCs), T cell subsets (memory CD8, CD4 and Treg cells) and "innate-like" T cell types, including subpopulations of γ/δ T cells and natural killer T (NKT) cells (30). Tissutal NK cells, similarly to other lymphocytes residing in tissues, may display markers such as CD69, CD103 (also known as αE integrin) and CD49a (also known as α1 integrin), which are functionally involved in retaining them in tissues and, hence, can be useful for the identification and isolation of tissue-resident (tr) NK cells (**Figure 1**). As discussed earlier, at least three-quarters of NK cells in non-reactive lymph nodes display a $CD56^{bright}$ $Perf^{low}$ phenotype (20, 31). This accumulation is compatible with the pattern of adhesion molecules (CD62L) and chemokine receptors (CCR7) expressed on circulating PB-$CD56^{bright}$ NK cells but not PB-$CD56^{dim}$ NK cells. From recent data, it is possible to speculate that a fraction of NK cells reaching the LN could be retained within the structure as trNK cells. Supporting this hypothesis is the presence of a distinct subset of NK cells in human SLTs characterized by co-expression of CD69 and CXCR6, high expression of CD54 (ICAM-1) but lacking CD117 (c-kit) and CD127, the latter specifically expressed by $CD56^{bright}$ NK cells (32). Because of the high level of CD54, these SLT-NK cells are also reminiscent of $CD56^{bright}$ $NKG2A^{pos}$ $CD94^{pos}$ $CD54^{pos}$ $CD62L^{neg}$ NK cells that accumulate in tonsils of EBV carriers, which produce high amount of IFNγ, show very low plasticity even after prolonged cytokine stimulation, and are able to potently restrict EBV-induced transformation of B cells (33).

Among solid tissues, liver is abundantly populated by NK cells, where they represent up to 30–40% of all the lymphocytes populating this organ (34). At steady-state, NK cells are preferentially located in the hepatic sinusoids, often adhering to the endothelial cells (35). Similar proportion of $CD56^{dim}$ and $CD56^{bright}$ NK cell populations have been reported to populate this organ (36), but only $CD56^{bright}$ has been described to own features of trNK cells. Indeed, liver $CD56^{bright}$ NK cells are characterized by higher level of EOMES transcription factor, expression of CXCR6 and CD69 as well as CCR5 but absence of CD62L and CCR7 (37). Interestingly, the expression of CD49e (also known as α5 integrin or VLA-5 α chain) has been recently identified has a reliable marker able to distinguish conventional "circulating" NK cells from *bona fide* liver-NK cells, which are otherwise negative for this marker (38). Many reports have suggested the importance of CCR5 and CXCR6 in their localization and retention within liver parenchyma, since their cognate ligands (CCL3, CCL5, and CXCL16) are constitutively expressed by various parenchymal and non-parenchymal cells in the liver, including cholangiocytes, sinusoidal endothelial cells, hepatocytes and Kupffer cells (34). Investigation of human liver transplants has indicated that $EOMES^{high}$ trNK cells can persist *in situ* for very long periods (up to 13 years in one human study), further supporting the idea that subsets of NK cells may stably reside within liver tissues. At the same time, circulating $CD56^{bright}$ $EOMES^{low}$ cells may be recruited to the liver and have the potential to become $CD56^{bright}$ $EOMES^{high}$ NK cells (39).

An exception to the aforementioned tissues is represented by lungs since: (i) the majority (∼80%) of NK cells populating these organs belongs to the $CD56^{dim}$ $Perf^{high}$ subset (40); (ii) only a limited fraction of Lung-NK cells is characterized by expression of markers consistent with tissue-residency (i.e., CD69). Interestingly, this fraction is mainly composed of $CD56^{bright}$ $CD16^{neg}$ and only a small proportion of $CD56^{dim}$ $CD16^{bright}$ NK cells (41), thus suggesting that "genuine" lung-resident NK cells may share some commonalities with $CD56^{bright}$ trNK cells found in the uterus, liver, and lymphoid tissues (37). Lung-NK cells were detected in the parenchyma only, and were not found outside of the parenchyma, (i.e., blood vessels or bronchi) (8, 41). Therefore, overall, these data support a model in which human lungs mainly contain highly differentiated NK cells recirculating between lung and blood, rather than a stable pool of tissue-resident NK cells (41). Consistent with this hypothesis, using a parabiotic mouse model, it has been recently shown that parabiont-derived donor NK cells are able to rapidly replenish the majority of NK cells in the lungs of recipient mouse (42).

Development of tissue-resident lymphocytes seems to involve a transcription program inducing the expression of genes involved in tissue-retention while inhibiting that of genes important for tissue egress and trafficking. In mice, it was recently described that the transcription factor Hobit (homolog of Blimp-1 in T cells or ZNF683), a zinc finger protein, acts in concert with Blimp-1 (B lymphocytes-induced maturation protein) to serve as a master regulator of tissue-residency for lymphocytes. Thus, Hobit and Blimp-1 mediate a common transcriptional program that is shared among tr memory (Trm) T cells, NKT, trNK cells, and helper-like ILCs. Together with Blimp-1, Hobit sustain unresponsiveness to signals for SLT re-circulation from peripheral tissues by suppressing expression of *S1pr1* (which encodes S1P1), *Sell* (which encodes CD62L) and *Ccr7* (which encodes CCR7) (30). The role of Hobit in human Trm cells is less clear. Recent reports have shown peculiar results with regard to the expression of Hobit/ZNF683 in the two major human PB-NK cell subsets. Indeed, Hobit has been detected at high levels in circulating $CD56^{dim}$NK cells (despite this transcription factor is almost absent in circulating NK cells in mice) while only poorly expressed by PB-$CD56^{bright}$ NK cells (43, 44).

However, it has been found that a strong Hobit/ZNF683 expression identifies a subset of intrahepatic $CD56^{bright}$ NK cells in human liver, which additionally express a distinct set of adhesion molecules (CD69, CD49a) and chemokine receptors (CXCR6) consistent with tissue residency (44). These data may suggest that Hobit expression in humans may instruct unique migratory properties in the two distinct circulating NK cell subsets. Whilst low expression of Hobit in circulating $CD56^{bright}$ NK cells could maintain high levels of CCR7 and CD62L

necessary for SLT entry, high level of Hobit in $CD56^{dim}$ and $CD56^{bright}$ trNK cells might down-regulate these markers on their surface, thus limiting their recirculation to SLT and tissue egress, respectively.

NK CELLS IN PREGNANCY

Pregnancy is a quite peculiar situation, in which an immunocompetent individual (the mother) is in contact for a long period of time with a genetically different immunodeficient individual (the fetus), and is characterized by a deep modification of mother's tissues. During the first trimester of pregnancy, extravillous trophoblast cells (EVT) from the fetus invade the maternal decidua penetrating through the basement membrane of the uterus epithelium with remodeling of the maternal spiral arteries. These changes ensure adequate nutrition of the fetus and are supported by immune cells present at the maternal-fetal interface (45). In normal pregnancy many different mechanisms exist to ensure tolerance of the semi-allogeneic fetus by the maternal immune defense, thus preventing fetus rejection and allowing the reproductive success.

The decidua is populated by a large variety of leukocytes, which represent approximately 30–40% of decidual cells. The most represented leukocyte populations are NK cells, $CD14^{pos}$ myelomonocytic cells and T lymphocytes (46). Decidual NK cells (dNK) represent 50–90% of total decidual lymphoid cells in the first trimester of pregnancy (47) (**Figure 1**). The number dwindles by the end of second trimester, and returns to basal levels at the end of pregnancy. NK cells have also been identified in non-pregnant endometrium (eNK) and their number changes throughout the menstrual cycle, reaching the maximal level in the post-ovulatory phase of the cycle (48). Most uterine NK cells do not express CD16 and show high levels of CD56. The dNK cells have been shown to exhibit unique phenotypic and functional properties. Indeed, relevant differences exist in the gene expression of the NK cell subsets present in peripheral blood and early pregnancy decidual tissues. CD9 tetraspanin, galectin, α-1 integrin and other adhesion molecules are overexpressed in dNK (49). Unlike resting PB NK cells, dNK cells express the CD69 marker and a large percentage express the NKp44 activating receptor. The expression levels of activating receptors/co-receptors (NKp46, NKp30, DNAM-1, NKG2D, and 2B4) are similar in dNK and PB NK cells and, regarding to inhibitory MHC class I-specific receptors, the dNK cells have been shown to express Killer Immunoglobulin receptor (KIRs), CD94/NKG2A and LILRB1 (also known as ILT2, LIR1, and CD85j). Interestingly, the KIR repertoire of dNK cells is skewed toward recognition of HLA-C, the only classical MHC Class I molecule expressed by trophoblast cells (46, 50). Although expressing both perforin and granzymes dNK cells are poorly cytotoxic, a characteristic that has been linked to the block in the polarization of cytolytic granules to the immunological synapse (51). Importantly, cytokines, such as IL-15 can restore the dNK cell cytotoxic function, a phenomenon that is crucial in normal pregnancy to control viral infection (52).

Various studies have shown peculiar functional capabilities of dNK cells. Indeed, they release a wide panel of cytokines, chemokines, and angiogenic factors that are involved in the development of placenta, tissue remodeling, trophoblast invasion and neoangiogenesis (48). Several studies analyzed the chemokine repertoire in endometrium and decidual tissues of women undergoing elective pregnancy termination, studying its involvement in NK recruitment. CXCL9 (Mig), CXCL10 (IP10), CXCL12 (SDF-1), CCL3 (MIP-1α) e CCL4 (MIP-1β) are constitutively expressed in the endometrium. First-trimester human trophoblast expressed and released chemokines able to exert their activity on NK cells, including CXCL12 and CCL3 (53). In line with these results studies have shown that chemokines produced by endometrial or trophoblast cells induce the peripheral blood NK cell chemotactic response. Decidual endothelial and stromal cells express CCL2 (MCP-1), CXCL8 (IL-8), CXCL10, CX_3CL1 (fractalkine), and CXCL12 while only stromal cells express detectable levels of CCL5 (Rantes) and CCL4. Noteworthy, CXCL10, CXCL12 and CX_3CL1 induce the migration of PB NK cell across primary cultures of decidual endothelial and stromal cells (54). Furthermore it has been shown that also chemerin is expressed in the uterus by EVT and stromal cells but not by decidual endothelial cells (DEC) (55–58). The treatment of DECs and stromal cells with progesterone enhanced CXCL10, CX_3CL1, and CCL2 but not CXCL12 levels, while estrogen treatment of stromal cells resulted in up-regulation of CXCL10 and CX_3CL1 (54–57). Moreover, the treatment of stromal cell primary cultures from pregnant, fertile non-pregnant, or menopausal women with progesterone and estrogen resulted in a significant up-regulation of chemerin secretion.

Although it is unclear how and when the various chemokines participate in the recruitment of dNK cells, it has been shown that dNK cells express high levels of CXCR3, low level of CXCR4 and very low levels of CXCR1, CXCR2, CX_3CR1 or CCR1, 2, 3, 5, 6, and 7. In this regard, CXCR3 and CXCR4 are involved in migration of decidual NK cells to CXCL9, CXCL10 and CXCL12 respectively (59). Moreover dNK cells migrate through stromal cells in response to CXCL10 and CXCL12 but not to CX_3CR1 (54). Interestingly, dNK cells from pregnant women express chemerin receptor (ChemR23 or CMKLR1) that induces their migration through stromal cells in response to chemerin. The different chemokine receptor profile between dNK and PB NK cells suggests that the phenotypic features of leukocytes recruited from peripheral blood during pregnancy can be influenced by the decidual microenvironment. In this regard, evidence indicates that the pregnant uterus is a good source of cytokines acting on NK cells including IL-15 (60). Interestingly *in vitro* culturing of PB NK cell with IL-2 or IL-15 induced a down-regulation of ChemR23 (18). In line with these observations studies have shown that co-culture of PB NK cells with stromal cells results in a chemokine receptor profile similar to that of decidual NK cells (54).

Nevertheless, it is noteworthy that the precise origin of dNK cells is not yet clear. It is possible to speculate that a pool of dNK cells may originate from PB NK cells recruited in decidua at early stages of pregnancy. On the other hand, studies suggest that they could also originate from *in situ* progenitor cells that,

in response to uterine stromal environment, differentiate into CD56^{bright} CD16^{neg} NK cells (61).

NK CELLS IN TUMOR TISSUES

A consolidated view considers NK cells as the more effective lymphocyte subset involved in immune surveillance of hematological malignancies, initial stages of solid tumors and blood spreading metastatic cells (62–64). Conversely, NK cells appear to be poorly efficient in controlling advanced, consolidated tumors due to different reasons, which comprise the plethora of immune suppressive factors characterizing the tumor microenvironment (63, 65). These include the expression by cancer cells of MHC class I molecules and immune checkpoint-ligands such as PD-Ls and B7-H3 (63, 66), the lack of expression or the release of soluble forms of ligands of activating receptors, and the presence of soluble immunomodulators, the prototypic one being represented by TGF-β1 (63, 65). Additional aspects impacting on the NK-mediated tumor immune surveillance are the low frequency and/or the quality of NK cells attracted in tumor tissues (**Figure 2**). Indeed, highly cytolytic CD56^{dim} CD16^{pos} NK cells are rare and immature CD56^{bright} CD16$^{low/neg}$ NK cells with low perforin content represent the majority of tumor-associated NK cells. Although some authors suggested the possibility of an *in situ* expansion of CD56^{bright} NK cells (67), a shared hypothesis considers as primum movens the type of chemokines/receptors interactions occurring in the tumor microenvironment.

The tumor orchestrates escape strategies and creates a chemokine milieu consisting of reduced expression of CXCL2, CX_3CL1, CXCL1, and CXCL8, attracting CD56^{dim} NK cells, and increased expression of CXCL9, CXCL10, CXCL19, and CCL5 that drives migration of CD56^{bright} NK cells. The dichotomy between high (CD56^{dim}) and low (CD56^{bright}) cytolytic NK cells has been widely studied and data show that pro-inflammatory cytokines can increase the killing properties of CD56^{bright} NK cells (68). However, this cytokine-mediated rescue mechanism might be deeply affected by TGF-β (69), which is highly represented in tumor tissues. This is because the tumor environment is rich in both TGFβ-1 producing cells and in factors that induce TGFβ activation, such as acidic pH, reactive oxygen species, proteases and specific members of integrin family (70). Active TGF-β1 decreases the expression of activating NK receptors and, by up-regulating mir27a-5p, of perforin 1 (Prf1) and granzyme B (GzmB), thus hampering NK cell cytotoxicity. Moreover, TGF-β1 might dampen CD56^{dim} recruitment and favor that of CD56^{bright} by modifying their respective chemokine receptor repertoires (13). In particular, TGF-β1 increases the expression of CXCR3 and CXCR4 in CD56^{brigh} and CD56dim NK cells, whereas, via mir27a-5p, down-regulates CX_3CR1 expression in CD56^{dim} cells (13, 71). CX_3CR1, whose cognate ligand is represented by CX_3CL1 (also known as fractalkine), is selectively expressed by CD56^{dim} NK cells and together with CXCR4 has been demonstrated to regulate NK cell-egress from bone marrow and NK cell extravasation (72). Interestingly, in agreement with this ability of tumors in inducing a regulatory milieu, an unusual low expression of CX_3CR1 has been reported in CD56^{dim} NK cell population of tumor-infiltrated bone marrow and peripheral blood of Neuroblastoma (NB) patients (13). Although a more detailed analysis should be performed to deepen whether this unusual chemokine receptor repertoire actually defines a peculiar CD56^{dim} population (73) mirroring the "broad spectrum of human Natural Killer Cell Diversity" (2), it is conceivable that CX_3CR1^{low} CD56^{dim} cells show defective migration toward tumor (or inflamed tissues). Conversely, the recruitment of CD56^{bright} NK cells in a CXCL9 and CXCL10 rich milieu might be favored by their constitutive expression of high levels of CXCR3 and CXCR4, which further increase under the influence of TGFβ-1 (8, 16, 17). Along this line, CD56^{bright} CD16^{low} represented the predominant NK cell population in the ascitic fluids of ovarian cancer patients (74). The concomitant up-regulation of CXCR3 and CXCR4 by TGF-β1 represents an interesting event if considering that these receptors are subject to cross regulation. Indeed, chemokine receptors' function can be modulated by desensitization, which is a physiological process that prevents overstimulation due to prolonged agonist exposure by signal attenuation or termination (27). Desensitization of a receptor can be dependent on its ligand (homologous desensitization) or by other ligands present in a complex chemokine gradient, a cross-desensitization called heterologous desensitization. In this context it has been shown that pre-stimulation of NK cells with CXCL9 inhibited NK cell migration not only to CXCR3 ligands but also to CXCL12, thus indicating that triggering of CXCR3 can promote both homologous and heterologous (CXCR4) desensitization (75).

In solid tumors a "fast track entrance" for CD56^{bright} CX_3CR1^{neg} CXCR3^{high} CXCR4^{high} NK cells might be the ectopic, neo-generated High Endothelia Venules (HEV) that contribute to the architecture of Tertiary Lymphoid Structures (TLS) (76, 77). These transient, un-capsulated lymphoid aggregates resembling Secondary Lymphoid Organs (SLO) have been detected in peri- or intra-tumor sites as well as in other chronic inflamed tissues. TLS share with SLO the presence of distinct T and B cell compartments, reactive Germinal Center (GC), Follicular Dendritic Cells (FDC), fibroblastic reticular cells (FRC) and lymphatic vessels, as well as HEV whose lining endothelial cells express highly specific addressin molecules, collectively termed peripheral node addressins (PNAd) (76). These are known to dictate adhesion and consequent extravasation of immune cells, including NK cells, within paracortical region of lymph nodes, an event that might occur also at TLS levels. In different tumors including lung, breast or gastrointestinal stromal tumors (GIST) (78), tumor-associated TLS might contribute to the preferential recruitment of CD56^{bright} NK cells that constitutively express the homing receptor CD62L and high levels of CCR7 specific for the lymph node chemoattractants CCL19 and CCL21. For example, in TLS associated to human lung cancer intra-tumoral PNAd^{+} HEV exclusively co-localized with CD62L^{+} lymphocytes (76). Notably, while TGF-β negatively impacts on CD56^{dim} NK cells recruitment in perturbed tumor tissues, upregulation of CCR7 may promote their migration to SLO and TLS. Accordingly, enrichment in CD56^{dim} CCR7^{+}

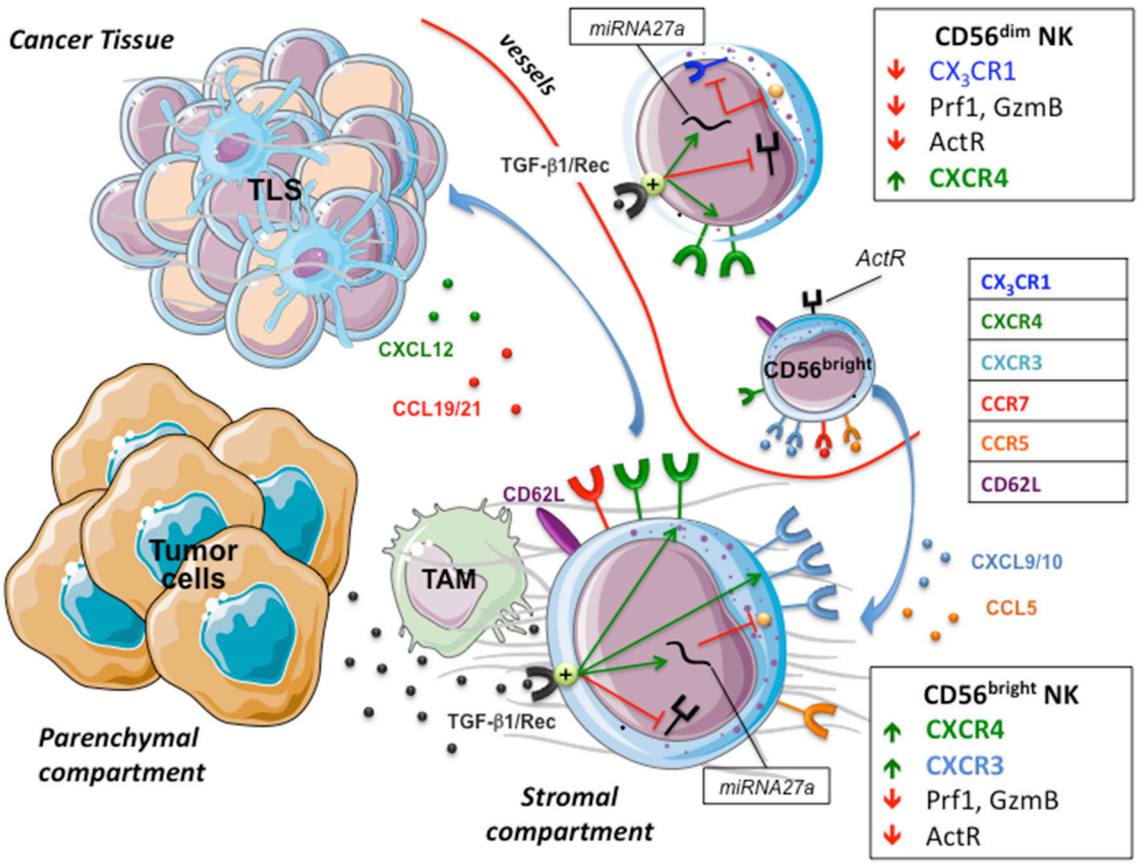

FIGURE 2 | NK cells in tumor microenvironment. The tumor chemokine milieu presents a reduced expression of chemokines attracting CD56dim NK cells, and an increased expression of CXCL9/10, CCL5, and CXCL19/21 that drives the migration of CD56bright NK cells toward the stromal compartment and tertiary lymphoid tissue (TLS). Tumor cells and tumor-associated macrophages (TAM) release/activate TGF-β1 that decreases the capability of NK cells to recognize and kill targets and modify their chemokine receptor repertoire, hampering the recruitment of CD56dim NK cells and favoring that of poor cytolytic CD56bright. ActR, Activating receptors; GzmB, Granzyme B.

KIR^+ $CD57^+$ highly cytotoxic NK cells has been documented in tumor-infiltrated lymph nodes of melanoma patients (79). Several mechanisms involved in the acquisition of CCR7 by $CD56^{dim}$ NK cells have been identified that include the crucial role of IL-18, highlighted by Mailliard et al. (80), and the possible uptake of CCR7 from surrounding cells by trogocytosis (81). Soluble IL-18 is produced by stimulated antigen presenting cells, in particular by macrophages that, as M2-polarized cells, might represent the most abundant immune population in the tumor microenvironment (82). Interestingly, a variable subset (30–40%) of unpolarized (M0) and M2 macrophages and most tumors associated macrophages (TAM) express a membrane form of IL-18 (mIL-18) (74, 83, 84). Upon TLR stimulation, macrophages polarize toward M1 and loose mIL-18, an event paralleled by the release of small amounts of soluble IL-18 (sIL-18) that, acting in close proximity, induces the expression of CCR7 in $CD56^{dim}$ NK cells (83). It is of note that, since M1 polarizing macrophages also acquire CCR7 expression (83), a contribution of trogocytosis-mediated uptake cannot be ruled out. Although mechanisms responsible for IL-18 membrane retention and release have to be clarified, this cytokine shows many predictable cleavage sites for extracellular proteases such as Matrix metallopeptidase (MMP) −2 and −9, which characterize the secretory profile of parenchymal tumor cells and TAM. Thus, also in the absence of pathogen-derived stimuli, the action of MMPs (or other still unknown mechanisms), may allow IL-18 shedding from TAM and the induction of CCR7 expression in $CD56^{dim}$ tumor-associated NK cells (TA-NK), thus promoting their migration to SLO and TLS.

In solid tumors CCR7 acquisition by NK cells may depend on close cell-to-cell contacts with macrophages or dendritic cells, whereas it is less plausible that tumor cells could play a relevant role. Indeed, TA-NK cells were found to be predominantly located in the stromal compartment, whereas they were rare/absent in the parenchyma in direct contact with tumor cells (40, 85). Regarding the compartmentalization of TA-NK cells, in an adenocarcinoma colon model, stromal-infiltrating NK cells had morphology compatible with actively migrating cells, and in some instances migrating NK cells co-localized with degraded matrix (85). In the same model,

most of the NK-poor tumor nodules were surrounded by a capsule-like structure with collagen IV and laminin, two major components of the basement membrane. On the contrary, tumor nodules lacking these containment structures were more infiltrated by NK cells. These observations, together with data showing that poor NK cells infiltration have been equally detected in both chemokines-rich and -poor tumors, strongly indicate stromal barriers as a hindrance impacting on possible NK-to-tumor cell contacts. Along this line, during imatinib mesylate therapy in GIST patients, the frequency of NK cells did not change in fibrous trabeculae, whereas significantly increased in the core of both localized or metastatic tumors, an observation that correlated with a better prognosis (78). Interestingly, a recent study analyzing the off-target effect of imatinib mesylate on immune cells showed that this drug causes a significant up-regulation of CXCR4 in both T and NK cells (86). Accordingly, NK cells *ex-vivo* isolated from peripheral blood of chronic myeloid leukemia patients receiving imatinib mesylate showed levels of CXCR4 significantly higher than those detected in healthy individuals (86). A study by Goda S. and colleagues (87) may in part explain how increased CXCR4 surface levels can facilitate NK cells to cross the bridge connecting the stroma and the tumor parenchyma compartments. In particular, they showed that human $CD56^{dim}$ $CD16^{pos}$ NK cell invasion into type I collagen is enhanced by CXCL12, the CXCR4 ligand, in a matrix metalloproteinases (MMP)-dependent manner. Notably, CXCL12 has been shown also to promote the production in monocytes (88) and megakariocytes (89) of MMP-9, which has protease activity on collagen IV. With this assumption, it is conceivable that therapies strengthening the CXCR4/CXCL12 axis could potentiate extracellular matrix degradation favoring NK (and T) cells migration toward tumor cells.

In light of these considerations, data on the NK cell phenotype and density in tumor sites cannot be considered "*per se*" a favorable prognostic factor and should be more and more integrated with data on NK cell localization with respect to stroma, parenchyma tumor cells and with the analysis of the whole immune landscape. For instance, high NK cell infiltration has been associated with improved survival in metastastic renal cell carcinoma but not in colorectal carcinoma (90). Contradictory results may depend on the method used to unequivocally identify NK cells, which still represents a major challenge as NKp46, the more reliable marker, is also expressed by other subsets of ILCs (91). Opposite clinical impact of NK cell infiltration in solid tumors might also depend on the targeted tissue, the tumor phase and the ratio between NK and tumor cell numbers. It has been demonstrated that NK cells can edit tumor cells modifying their immunogenicity. In particular, in NK and melanoma cell co-cultures performed at low effector/target ratios, which reflect the level of NK cell infiltrates observed at the tumor site, an initial tumor cell lysis is followed by an equilibrium phase characterized by decreased susceptibility to killing due to up-regulation of both classical and non-classical MHC class I molecules on melanoma cells. This effect is mediated by IFN-γ released by NK cells activated upon melanoma cell recognition. Importantly IFN-γ and TNF-α are also potent inducers of the expression of the immune checkpoint ligands PD-L1 and PD-L2 in macrophages/dendritic cells, tumor cells and tumor-associated endothelial cells (92, 93). Moreover, TNF-α is known to promote Epithelial-Mesenchymal Transition (EMT), a process leading epithelial tumors to acquire a less differentiated, pro-metastatic phenotype. Along this line, in lung cancer, a recent report showed an important correlation between PD-L1 expression and EMT score (93, 94). Thus, low number of NK cells contacting tumor cells might have more undesirable than beneficial effects, being unable to efficiently eliminate tumor cells while causing a gradual accumulation of cytokines that exert a paradoxical tumor promoting effect by modifying the immunogenicity of tumor cells.

Whatever the case, when designing NK cell-based immunotherapeutic approaches for cancer patients, we should take into account the relevance of the molecular mechanisms regulating NK cell migration into tumors. For instance, a recent and promising approach is represented by the infusion of NK cells engineered to express chimeric antigen receptor (CAR) specific for tumor-associated antigens (65, 95, 96). The efficacy of adoptively transferred CAR-NK might be deeply limited by their inability to cross stromal barriers and to adhere to parenchymal tumor cells, as recently suggested for T cells by Caruana and colleagues (97). It was pointed out that *in vitro* manipulation aimed to the CAR engineering of T cells leads to silencing of heparanase (HPSE), an endoglycosidase that cleave heparan sulfate proteoglycans of ECM, thus reducing the invasive potential of CAR-T cells in solid tumors. Thus, cell-based therapy may also include strategies to favor migration of effector cells through stromal compartment and tumor parenchyma, a phenomenon unlikely to occur, particularly in advanced solid tumors.

NK CELLS IN INFLAMED TISSUES

The perturbation mediated by pathogens in peripheral tissues results in the early activation of resident or recruited cells of the innate immunity with a consequent boost of chemotactic factors, which attract different immune cells including peripheral blood mature conventional $CD56^{dim}$ NK cells. These cells mainly differentiate in the bone marrow and express CXCR1, CXCR2, chemR23, S1P5, CXCR4, and CX_3CR1 (16, 17, 26). This chemokine receptor repertoire drives NK cells to inflamed tissues. Importantly, the relative expression of CXCR4 and S1P5 in developing mouse NK cells has been described to regulate bone marrow egress into circulation (98). Moreover, in bone marrow, prevalent CX_3CR1 expression by $KLRG1^+$ NK cells located in sinusoids suggested its crucial role for NK cell entry into the vascular compartment (99).

The presence of NK cells in healthy and inflamed peripheral tissues (18, 26, 29) has been well documented and different studies demonstrated the existence of a crucial crosstalk between $CD56^{dim}$ NK cells and DC or macrophages (**Figure 3**). NK/DC interactions resulted in a bidirectional activation leading to killing of immature DC (iDC) by autologous $NKG2A^+$ KIR^{neg}

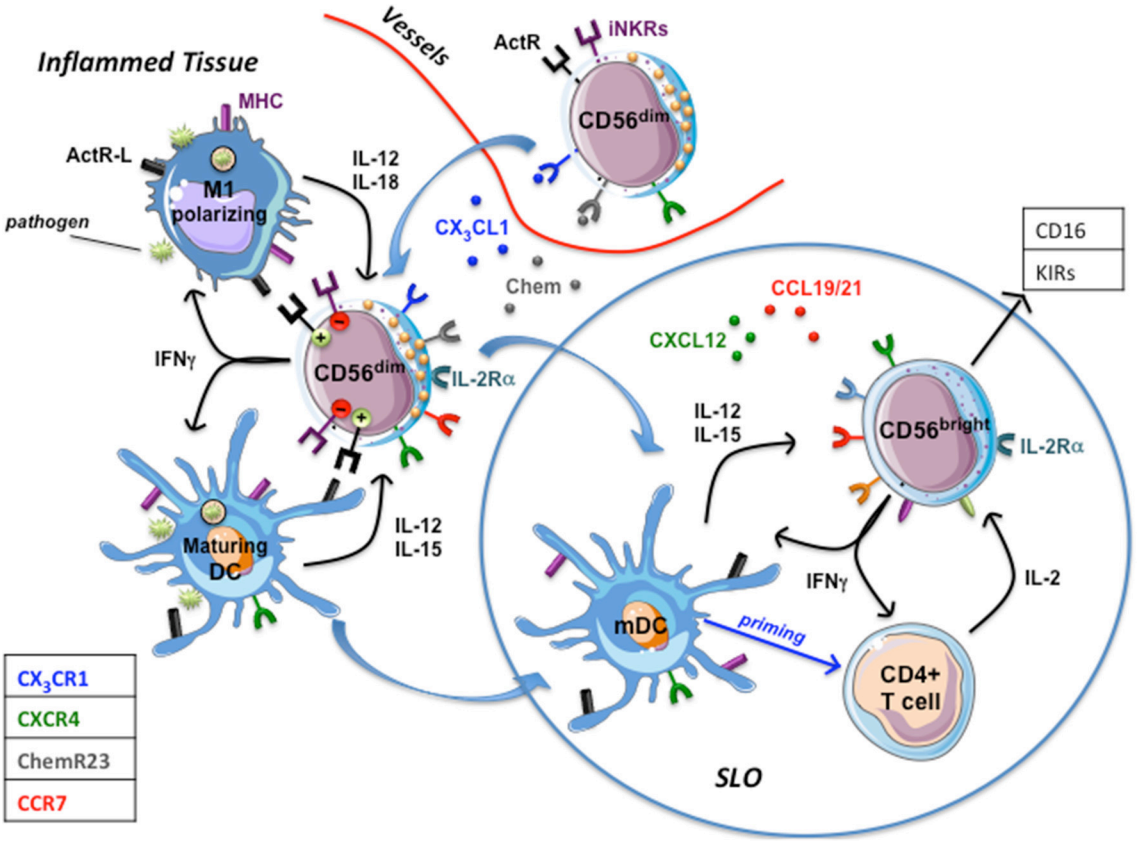

FIGURE 3 | NK cells in inflamed tissues. Inflamed tissue produce chemokines (Chemerin, CX3CL1) that drive the migration of circulating CD56dim prf1high NK cells. Once in tissues they interact with DC and macrophages that, upon pathogen recognition, have begun to mature (mDC) or polarize toward a proinflammatory functional phenotype (M1). Maturing DC and M1-polarizing macrophages release immunostimulatory cytokines that induce NK cells to produce large amounts of IFN-γ (which potentiate phagocytes' functions) and to express IL-2Rα and CCR7, which drive their migration into secondary lymphoid organs (SLO). mDC migrated to SLO and DC-primed T cells producing IL-2 stimulate CD56dim NK cells and CD56bright NK cells that acquire a $CD16^{pos}$ KIR^{pos} phenotype. ActR-L, Activating receptors ligands; iNKRs, MHC class I specific inhibitory receptors (KIRs, CD94/NKG2A); Chem, chemerin.

NK cells. NK-mediated DC lysis, due to a pivotal role of NKp30 and DNAM-1 activating receptors (20, 100), is restricted to iDC undergoing an unfruitful maturation process, characterized by an inadequate MHC class I up-regulation. This mainly impacts on the expression of the non-classical HLA-E molecule, the cognate ligand of the CD94/NKG2A inhibitory receptor. This effect has been interpreted as a negative selection strategy aimed to avoid inappropriate antigen presentation by MHC class I low DCs, which could result in tolerogenic responses. DC that underwent an appropriate maturation program (mature dendritic cells, mDC) are spared from NK-cell mediated attack since they express very high levels of classical MHC class I and significantly up-regulated HLA-E (101, 102). The principal NK cell-derived mediators shaping DC immune-phenotype are represented by TNFα and IFNγ, whose release depends on the synergistic activity of IL-12 and IL-15 produced by pathogen stimulated DC. mDC *de novo* express CCR7, (a phenomenon also occurring during pathogen-driven macrophage polarization toward M1) which confers to these cells the competence for migrating to SLO. In SLO T cell zone, DCs co-localize with NK cells belonging to the $CD56^{bright}$ $CD16^{low/neg}$ subset, which constitutively express CCR7 (101).

The complex interactions among NK cells expressing IL-2Rα (CD25), DC-primed T cells producing IL-2 and mDC result in a conspicuous IFN-γ production by NK cells shaping T cell priming, polarization and adaptive immune responses (101). Whether, in SLO, NK cells may also shape macrophages' functions remains to be elucidated. $CCR7^{pos}$. NK cells can migrate to SLO via HEV since they also express high levels of CD62L. However, a predominant population of $CCR7^{pos}$ $CD56^{bright}$ $CD16^{neg}$ NK cells has been described in seroma fluid, thus depicting afferent lymph as an alternative way for $CD56^{bright}$ NK cells to colonize SLO (8). Interestingly, it has been observed, in seroma, the presence of low numbers of $CD56^{pos}CD3^{neg}$ cells expressing CX_3CR1, KIRs and CD16 molecules, a phenotype usually characterizing classical $CD56^{dim}$ NK cells (8). Although a multiparametric analysis providing information about a possible co-expression of CCR7 was lacking, these data support the hypothesis that, *in vivo*, cytolytic $CD56^{dim}$ NK cells might also migrate to "perturbed" SLO. Along this line,

the interaction of $CD56^{dim}$ NK cells with M0 or M2 macrophages polarizing toward M1 upon TLR engagement results in the acquisition of CCR7 and of a fully activated NK cell status characterized by high CD69 and IL2Rα expression, release of large amount of IFN-γ and increased cytolytic activity (83). Thus, in inflammatory conditions, M1-activated $CD56^{dim}$ NK cells, becoming competent for SLO migration thanks to the acquisition of CCR7, might deeply contribute to both immunosurveillance of tumor metastases and control of infected cells. Migration of fully functional $CD56^{dim}$ NK cells to SLO, could be particularly relevant in the context of KIR/KIRL-mismatched haploidentical stem cell transplantations (haplo-HSCT). Indeed, in SLO, NK-mediated killing of recipient mDC and residual T cells might contribute to the low rate of graft vs. host disease (GVHD) and graft rejection documented in this clinical setting (103).

Nevertheless, is there any *in vivo* evidence that $CD56^{dim}$ NK cells might traffic through and leave SLO, thus recirculating via efferent lymph? A few preliminary reports indicate this possibility. Non-reactive LNs or LNs characterized by sinus hyperplasia lack or show low expression of $KIR^{pos}CD16^{pos}$ cells. Interestingly, reactive LNs characterized by paracortical/follicular hyperplasia harbor a significant percentage of cells expressing KIR and CD16 and a similar $KIR^{pos}CD16^{pos}$ cells enrichment was observed in the efferent lymph (i.e., toracic duct). Several observations, including a difference in the telomerase length, strongly suggest that $CD56^{bright}$ $CD16^{neg}$ KIR^{neg} cells can acquire a KIR^{pos} $CD16^{pos}$ phenotype thanks to the influence of the different pro-inflammatory cytokines present in LNs (28). However, the hypothesis that $CD16^{pos}$ KIR^{pos} NK cells might migrate to and expand in LNs before egressing via efferent lymph cannot be ruled out. In this context, in pathogen-perturbed tissues, $CD56^{dim}$ NK cells interacting with macrophages acquire the competence to SLO migration and, expressing high levels of IL2Rα (83), become highly responsive to IL-2 produced by T cells in the paracortex area of LN.

Pro-inflammatory cytokines are capable of shaping innate and adaptive immune responses also acting on the establishment of the NK cell memory reservoir. Both in mouse and human, it has been described that the CMV-driven onset of memory NK cell populations requires the presence of pro-inflammatory cytokines such as IL-12 and IL-18. Cytokines represent the third signal essential to generate, expand and recall NK cell memory. Signal 1 is represented by receptor-mediated antigen recognition, LY49D in mouse and NKG2C or KIR2DS1 in humans, and signal 2 by co-stimulatory signals, DNAM-1 and CD2 in mouse and human, respectively (15, 104). In addition, cytokines by themselves are capable of generating memory-like NK cells in an antigen–independent setting (14), as NK cells, shortly cultured in the presence of IL-12, IL-15 and IL-18, showed superior IFN-γ and TNF-α production and cytotoxicity in response to tumor targets and conferred more protection to leukemia or melanoma in xenograft mouse models. Thus, full NK cell activation and antigen-dependent or -independent generation of NK cell memory requires cytokines-mediated signals. It should be considered that cytokines also strongly impact on the chemokine receptor repertoire of NK cells. Beside sIL-18 whose capability of inducing CCR7 expression has been discussed above, IL-15 has been shown to down-regulate CX_3CR1 expression in mouse bone marrow-derived NK cells (10) and in human PB NK cells (12), thus reducing the chemotactic response to CX_3CL1 ligand (12). IL-12 in association with IL-2 significantly decreased the CXCR3 mRNA and their surface expression in NK cells (9). Additionally, IL-2 alone has been shown to down-regulate the surface expression of CXCR1 as well as of CXCR4 inhibiting NK cell migration in response to CXCL12. On the other hand, IL-2 up-regulated the surface expression of CXCR3 increasing NK cell migration in response to its ligands CXCL9 and CXCL10 (11).

Regarding the migratory properties of memory NK cells, different questions remain unanswered. Do cytokines that drive their onset, impact on their chemokine receptor repertoire contributing to the generation of tissue-resident memory NK cells in various anatomical areas? Does the maintenance of the NK cell memory pool involve tissue-restricted reactivation of resident memory NK cells or do these cells maintain the potential to recirculate? Studies focused on mouse recall response to haptens provided some relevant indications. These studies showed that memory NK cells responsible for the immune response were the $CD49a^{+}$ $DX5^{neg}$ liver resident NK cells, and that the activity of CD18 and P-selectin, molecules involved in trafficking of NK cells, was needed (15). In this scenario, the characterization of human memory NK cells in terms of chemokine receptor expression, before and after cytokine-stimulation, could be particularly relevant.

CONCLUDING REMARKS

It has become evident that NK cells are not constituted by a homogeneous population of innate lymphocytes but rather by different subtypes with specific abilities as well as distinct homing properties. Investigating how NK cell subsets distribute in human body has relevance not only for a better understanding of our immune defenses but also for exploiting these cytotoxic cells in therapeutic settings.

It is worth noting that the migratory properties of NK cell subsets are relevant not only for identifying the region in which they should exert their activity. Recent reports indicate that NK cells could acquire specific properties, such as cytotoxicity, only upon their migration to secondary lymphoid organs where the cytokine milieu would induce their further differentiation. At the same time, although the picture of tissue-resident NK cells is still fuzzy, it is conceivable that these subsets of NK cells might locally acquire peculiar properties, such as release of specific soluble factors able to affect their properties but also influence other cells present in the microenvironment. As a matter of fact, NK cells (as well as all other innate lymphoid cells of which they represent the prototype) are more and more emerging as accessory cells able to modulate the functions of neighboring cells, including antigen presenting cells, in the environment in which they are attracted/hosted.

Despite the relevance of these issues, several open questions still remain to be addressed regarding the ability of NK cells

to infiltrate and reside in either healthy or pathological tissues. Decades after the discovery of NK cells as lymphocytes able to recognize and kill cancer cells without prior sensitization to them, we still miss a clear and complete depiction of the phenotype and properties of tumor-infiltrating NK cells. Similarly, although a number of studies have now highlighted the relevance of NK cells in the control of viral infections, how these cytotoxic lymphocytes recirculate and/or are retained in infected tissues still remain to be clearly determined, at least in humans.

On the other hand, novel technologies allowing extensive multiparametric analyses, either by mass cytometry or classical flow cytometry, not even conceivable until only a few years ago, might now open new avenues for a comprehensive mapping of tissutal NK cells. The path appears already tracked since we have now, as reported in the present review, a better appreciation of at least some of the molecules and the signaling ruling the homing properties of these innate lymphocytes.

AUTHOR CONTRIBUTIONS

RC, PC, AD, GF and CB wrote the manuscript. FB, BC and SR read the manuscript and provided critical input.

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Imatinib and Nilotinib Off-Target Effects on Human NK Cells, Monocytes, and M2 Macrophages

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Tyrosine kinase inhibitors (TKIs) are used in the clinical management of hematological neoplasms. Moreover, in solid tumors such as stage 4 neuroblastomas (NB), imatinib showed benefits that might depend on both on-target and immunological off-target effects. We investigated the effects of imatinib and nilotinib on human NK cells, monocytes, and macrophages. High numbers of monocytes died upon exposure to TKI concentrations similar to those achieved in patients. Conversely, NK cells were highly resistant to the TKI cytotoxic effect, were properly activated by immunostimulatory cytokines, and degranulated in the presence of NB cells. In NB, neither drug reduced the expression of ligands for activating NK receptors or upregulated that of HLA class I, B7-H3, PD-L1, and PD-L2, molecules that might limit NK cell function. Interestingly, TKIs modulated the chemokine receptor repertoire of immune cells. Acting at the transcriptional level, they increased the surface expression of CXCR4, an effect observed also in NK cells and monocytes of patients receiving imatinib for chronic myeloid leukemia. Moreover, TKIs reduced the expression of CXCR3 (in NK cells) and CCR1 (in monocytes). Monocytes also decreased the expression of M-CSFR, and low numbers of cells underwent differentiation toward macrophages. M0 and M2 macrophages were highly resistant to TKIs and maintained their phenotypic and functional characteristics. Importantly, also in the presence of TKIs, the M2 immunosuppressive polarization was reverted by TLR engagement, and M1-oriented macrophages fully activated autologous NK cells. Our results contribute to better interpreting the off-target efficacy of TKIs in tumors and to envisaging strategies aimed at facilitating antitumor immune responses. ***The Journal of Immunology*****, 2017, 199: 1516–1525.**

Virtually all patients with chronic myeloid leukemia (CML) and 20% of adult patients with acute lymphoblastic leukemia carry the translocation between the long arms of chromosomes 9 and 22 that generates the Philadelphia chromosome with a chimeric gene that encodes the fusion protein BCR-ABL, having constitutively activated tyrosine kinase activity. Imatinib mesylate (Glivec) was the first designed protein tyrosine kinase inhibitor (TKI) used in the treatment of CML and BCR-ABL$^+$ ALL (1–3). This drug binds to the protein in a fashion that prevents ATP interacting with the ATP-binding site of the ABL kinase, thereby blocking the tyrosine phosphorylation of proteins involved in BCR-ABL signal transduction (4). Importantly, in imatinib-resistant or intolerant BCR-ABL$^+$ patients, an alternative therapy is based on the administration of nilotinib (Tasigna), a second-generation TKI that, in vitro, is more potent and selective than imatinib as a BCR-ABL tyrosine kinase inhibitor (5–7).

Imatinib and nilotinib also target the discoidin domain receptors 1 and 2, the platelet-derived growth factor receptors α and β (8), the M-CSF receptor (9), and KIT (stem cell growth factor receptor, CD117) (10, 11). KIT is constitutively active in most human gastrointestinal stromal tumors (GISTs), a common mesenchymal neoplasm of the gastrointestinal tract and, in this context, imatinib is registered for the treatment of GISTs (12, 13). Neuroblastomas (NB) represent the most common extracranial solid malignancy of childhood (14); they frequently localize in the abdomen, particularly in the adrenal gland or at lumbar sympathetic ganglia. Metastatic disease (stage 4), very often characterized by bone marrow (BM) infiltration, is diagnosed in at least 50% of NB patients and it is still associated with a dismal prognosis because of a high risk of tumor relapse. Children with either refractory or relapsing metastatic NB have been recently enrolled in a two-stage, phase II clinical trial with imatinib; the drug was well tolerated and showed benefits in a subset of patients, particularly those with BM as the only site of metastatization, low tumor infiltration, and low imatinib exposure (15).

It has been suggested that the efficacy of imatinib in GISTs might depend on both on- and off-target effects, with the latter being

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Abbreviations used in this article: BM, bone marrow; CML, chronic myeloid leukemia; GIST, gastrointestinal stromal tumor; mIL-18, the membrane-bound form of IL-18; NB, neuroblastoma; PB, peripheral blood; TKI, tyrosine kinase inhibitor.

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related to the antiangiogenic effects and strengthening of antitumor immune responses (16–18). It is conceivable to speculate that also in NB, the efficacy of imatinib might be at least in part related to the enhancement of the anti-NB activity of immune cells, such as NK cells. When properly activated, NK cells exert a potent cytolytic activity and release immunostimulatory cytokines such as IFN-γ that potentiate both innate and adaptive immune responses, thus representing important effectors against both hematological and nonhematological malignancies (19, 20).

The NK cell activation depends on the interaction between activating NK receptors and their cognate ligands on tumor targets. In humans, the principal activating receptors include NKp46, NKp30, and NKp44 (collectively termed natural cytotoxicity receptors), DNAM-1, and NKG2D (21, 22). Different ligands have been identified that are upregulated or de novo expressed on the surface of tumor cells. Whereas PVR and nectin-2 are ligands of DNAM-1 (23, 24), and NKG2D interacts with the stress-inducible molecules MICA/B and ULBPs (25), the ligands of natural cytotoxicity receptors are not yet fully defined. NKp46 is still an orphan receptor, whereas B7-H6 (26) and a novel isoform of the mixed-lineage leukemia protein (MLL5) (27) have been identified as ligands of NKp30 and NKp44, respectively. NK cell activity unleashed by the interaction between activating receptors and their ligands is under the control of receptors that recognize optimal levels of self HLA class I molecules on potential targets and transduce inhibitory signals (21, 28). These NK inhibitory receptors include the killer Ig-like receptors (CD158) and the CD94/NKG2A heterodimer.

Either downregulation or loss of HLA class I molecules, which frequently occur in various types of malignancies, including NB, allows an efficient NK-mediated killing of tumors (29). However, it has been shown that several mechanisms might operate in the tumors to *escape* from immune-mediated surveillance (14, 30). The antitumor activity of NK cells depends on their capability of reaching and infiltrating the tumor tissues. NK cell migration in pathological tissues must be driven by chemokine gradients that are sensed through the expression of chemokine receptors that include CXCR3 (CXCL4, 9, 10, 11 receptor), CXCR1 (CXCL8 receptor), CX_3CR1 (CX_3CL1 receptor), and CXCR4 (CXCL12 receptor). The latter drives NK cells toward peripheral tissues and is essential for homing and maintenance of NK cells in stromal niches within the BM (31). Upon reaching the tumor microenvironment, NK cells encounter not only neoplastic cells, but also other immune effectors such as macrophages, which are the most represented leukocytes in cancer tissues. Peripheral blood (PB) monocytes express CCR1, are recruited by CCL1 (MIP-1α), and differentiate into macrophages (M0) under the influence of M-CSF (also known CSF1) (32). M0 cells can polarize toward either the M1 immunostimulatory or the M2 "alternative" immunosuppressive functional phenotype, depending on microenvironmental conditions.

The tumor microenvironment has been shown to promote M2 polarization that is characterized by high expression of scavenger receptors, such as CD206, and the release of factors that promote, instead of suppress, tumor growth. These include vascular endothelial growth factor, fibroblast growth factor, matrix metalloproteinases, and immunomodulatory cytokines, such as IL-10 and TGF-β, which in NK cells affect the expression of activating receptors and modulate the chemokine receptor repertoire (33). Importantly, the M2 status is not definitive, and TLR engagement can promote M1 polarization of macrophages, which release high amounts of proinflammatory/immunostimulatory cytokines, such as IL-1β, IL-6, TNF-α, IL-12, and IL-18 and are capable of inducing strong activation of NK cells (34, 35).

In this study, we examined the effect of two TKIs, imatinib and nilotinib, routinely used in the clinical management of several neoplasms, on the survival and function of human NK cells, monocytes, and macrophages, also considering their polarization properties and ability to interact with autologous NK cells.

Materials and Methods

Cells and TKIs

Buffy coats were collected from healthy volunteer blood donors admitted at the blood transfusion center of IRCCS S. Martino-IST after obtaining informed consent, and the study was approved by the Ethics Committee of IRCCS San Martino-IST (39/2012). After standard Ficoll-Paque density-gradient (Euroclone, Pero, Italy), monocytes and NK cells were purified with human monocyte cell isolation kit II or a human NK cell isolation kit, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany). Macrophages (M0) were obtained culturing monocytes for 7 d in 24 lumox multiwell TC-QUALITAET plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 5×10^5/ml and in the presence of 100 ng/ml rM-CSF (PeproTech, London, U.K.). To obtain M2 macrophages (M2), M0 were cultured for an additional 18 h with 20 ng/ml rIL-4 (PeproTech). For coculture experiments, NK cells, simultaneously purified with monocytes, were cultured for 7 d with recombinant human IL-2 (32 U /ml) (Proleukin; Chiron, Emeryville, CA), then harvested, washed, and added to autologous macrophages. To obtain short-term activated NK cells, purified NK cells were cultured overnight with rIL-12 (1 ng/ml) (PeproTech) and rIL-18 (Medical and Biological Laboratories, Nagoya, Japan) at 100 or 20 ng/ml for IFN-γ production or a CD107A degranulation assay, respectively. For TLR stimulation, macrophages were incubated for 24 h with 100 ng/ml LPS from *Escherichia coli* (Sigma-Aldrich) either in the absence or in the presence of imatinib or nilotinib. M2 macrophages were cocultured overnight with autologous NK cells in the presence of LPS at the 1:1 NK cell/macrophage ratio either in the absence or in the presence of drugs. For macrophage differentiation experiments, monocytes were cultured for 7 d with M-CSF in the absence or in the presence of imatinib or nilotinib, which were added to the cultures daily.

The NB cell line HTLA-230 was provided by Dr. E. Bogenmann (Children's Hospital Los Angeles, Los Angeles, CA) (36); the SH-SY5Y cell line was purchased from Banca Biologica and Cell Factory (IRCCS Azienda Ospedaliera Universitaria San Martino-IST, Genoa, Italy). NB cell lines were cultured in the presence of RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Biochrom, Berlin, Germany), 50 mg/ml streptomycin, 50 mg/ml penicillin (Sigma-Aldrich), and 2 mM glutamine (EuroClone). NB cell lines were periodically checked for *MYCN* amplification by fluorescence in situ hybridization analysis and for morphology, proliferation rate, and mycoplasma contamination, after thawing and within four passages in culture.

The K-562 (chronic myelogenous leukemia) cell line (American Type Culture Collection, Rockville, MD) was maintained in RPMI 1640 medium with 10% FBS (Biowest, Nuaille, France).

Clinical-grade imatinib mesylate (Glivec) and nilotinib (Tasigna) were provided by Novartis Pharma (Basel, Switzerland) to M.V.C. (MTA number 39985). The lyophilized powders were reconstituted in distilled water (imatinib) or DMSO (nilotinib) at the concentration of 10 mg/ml and further diluted in cell culture medium at the indicated work concentrations and times. Medium supplemented with the same percentage of DMSO present at the various nilotinib concentrations was used as a control.

NB and CML patients

The PB samples were collected after informal consent from seven patients diagnosed with high-risk NB either at the disease onset or relapsing (admitted at the Oncology Units of the Istituto Giannina Gaslini, Genoa and Ospedale Bambino Gesù, Rome), two pediatric CML patients (pCML1 and pCML3) (admitted at Ospedale Bambino Gesù, Rome), and three adult CML patients (aCML2, aCML3, and aCML4) (admitted the Hematology Unit, University of Pavia, Pavia). The CML patients were taking imatinib therapy daily.

After standard Ficoll-Paque density gradient separation, PBMCs were treated or not with rIL-12 (1 ng/ml) and rIL-18 (100 ng/ml) in the presence or in the absence of imatinib (6 μg/ml) for 24 h. For the analysis of NK cells and monocytes, PBMCs were stained with a mixture of anti–CD56-PC5 and anti–CD3-FITC (NK cells) and anti-CD14 (monocytes) (Beckman Coulter/Immunotec, Marseille, France). PBMCs derived from healthy volunteer blood donors were used as control.

Flow cytofluorimetric analysis and cytokine release assay

For cytofluorimetric analysis (FACSCalibur; Becton Dickinson, Mountain View, CA), cells were stained with PE-, FITC-, or PC5-conjugated mAbs or with unconjugated mAbs followed by PE-conjugated isotype-specific goat anti-mouse second reagent (SouthernBiotech, Birmingham, AL). Isotype-matched irrelevant mAbs were used as control. Monocytes and macrophages were preincubated for 30 min at 4˚C with FcR blocking reagent (Miltenyi Biotec) before specific mAb staining. For apoptosis and necrosis assays, before cytofluorimetric analysis, cells were incubated for 10 min at room temperature with annexin V and TO-PRO-3 iodide (Life Technologies, Carlsbad, CA) to identify apoptotic (annexin V^+), necrotic (TO-PRO-3 $iodide^+$), or viable (annexin V^-, TO-PRO-3 $iodide^-$) cells. For phagocytosis assays, macrophages were incubated with unopsonized pHrodo *E. coli* BioParticles or opsonized pHrodo *E. coli* BioParticles (Life Technologies), at the 1:5 macrophage/BioParticle ratio, at the indicated times and then harvested and analyzed by flow cytometry. On every experimental session, the flow cytometer performances were monitored and the reproducibility of the fluorescence intensity was aligned by calibrated microsphere (Becton Dickinson).

Cytokine content in the supernatants of NK cells or macrophages was quantified by ELISA kits: IFN-γ, TNF-α, IL-12p40/p70 (Life Technologies), and IL-18 (Medical and Biological Laboratories).

CD107a degranulation assay

HTLA-230 or cytokine-activated NK cells were cultured for 24 h in medium alone or supplemented with different concentrations of imatinib or nilotinib. Then cells were cocultured for 3 h at the E:T ratio of 1:1 in the presence of anti–CD107a-PE mAb. M2 macrophages were cocultured for 24 h with autologous NK cells in the presence of LPS at the 1:1 NK/macrophage ratio either in the absence or in the presence of drugs. NK cells were then collected and incubated with the HTLA-230 cell line at the E:T ratio of 1:1 for 3 h in the presence of anti–CD107a-PE mAb. Before flow cytometry, NK cells were stained with a mixture of anti–CD56-PC5 and anti–CD3-FITC.

PBMCs derived from CML patients, treated or not with I6, or PBMCs derived from healthy donors were cocultured with the K562 cell line for 3 h in the presence of anti–CD107a-PE mAb, taking in consideration the percentage of NK cells and using a NK/target ratio of 1:1. Before flow cytometry, NK cells were stained with a mixture of anti–CD56-PC5 and anti–CD3-FITC.

Real-time PCR

Total RNA was extracted from NK cells, monocytes, and SH-SY5Y cells using the miRCURY RNA isolation kit—cell and plant (Exiqon), according to the manufacturer's guidelines. RNA (300 ng) was reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen). Real-time PCR was performed using specific TaqMan gene expression assays (Applied Biosystems). CXCR4 gene expression was normalized to HPRT1 gene expression. Experiments were performed in triplicate.

Antibodies

The following mAbs were produced in our laboratory: BAB281 (IgG1, anti-NKp46), AZ20 (IgG1, anti-NKp30), Z231 (IgG1, anti-NKp44), BAT221 (IgG1, anti-NKG2D), KRA236 (IgG1, anti-DNAM-1), MA127 (IgG1, anti-NTBA), PP35 (IgG1, anti-2B4), C227 (IgG1, anti-CD69), MAR93 (IgG1, anti-CD25), M5A10 (IgG1, anti-PVR), L14 (IgG2a, anti–Nectin-2), 5B14 (IgM, anti–4IgB7-H3), BAM195 (IgG1, anti-MICA), A6136 (IgM) and 6A4 (IgG1) (anti–HLA class I), D1.12 (IgG2a anti–HLA-DR), and C127 (IgG1 anti-CD16). Anti–PD-L1.3.1 (IgG1, anti-PD-L1) and anti–PD-L2 (IgG1, anti–PD-L2) mAbs were produced in D. Olive's laboratory. Anti-CD14 (IgG2a) and a mixture of anti–CD56-PC5 and anti–CD3-FITC (IgG1) and anti–CD20-FITC were purchased from Beckman Coulter/Immunotec; anti-CD64 (IgG1), anti–CD80-PE, anti–CD107A-PE, and anti–CD206-FITC were purchased from BD Biosciences (San Diego, CA); anti–human IL-18 (IgG1) and anti–CX3CR1-PE (rat, IgG2b) were purchased from Medical and Biological Laboratories; anti-CCR1 (IgG2b), anti-CCR7 (IgG2a), anti-CXCR4 (IgG2b), anti-CXCR3 (IgG1), anti–CD204-PE, and anti–M-CSFR (IgG1) were purchased from R&D Systems (Minneapolis, MN); anti–CD31-PE, anti–CD33-PE, anti–CD36-PE, and anti–CCR6-PE were purchased from Miltenyi Biotec; anti-CXCR1 (IgG1) and anti-ULBP3 (166510, IgG2a) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); and anti–annexin V-FITC was purchased from eBioscience (San Diego, CA). All Abs were of mouse origin, unless otherwise specified. PE and FITC isotype-matched mouse (BD Biosciences) (Miltenyi Biotec) or rat (Medical and Biological Laboratories) mAbs were used as negative controls.

Statistical analysis

A Wilcoxon–Mann–Whitney p value test (nonparametric significance test) was employed. Graphic representation and statistical analysis were performed using PASW Statistics version 20.0 software (formerly SPSS Statistics) (IBM, Milan, Italy) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Results

PB NK cells are more resistant than monocytes to the cytotoxic effects of imatinib and nilotinib

The HTLA-230 human NB cell line was treated with concentrations of either imatinib or nilotinib ranging from 120 to 0.3 μg/ml (Fig. 1A, Supplemental Fig. 1A) and after 24 h assessed for apoptosis and/or necrosis by staining with annexin V and TO-PRO-3 iodide and flow cytometry analysis. Whereas the cells were unaffected by nilotinib, with imatinib concentrations ≥15 μg/ml, increasing numbers of necrotic cells were detected and all cells died upon exposure to concentrations of 120 μg/ml.

We analyzed the influence of decreasing concentration of drugs on the survival of circulating monocytes and NK cells. Monocytes and NK cells were purified from PB of healthy donors and their viability was assessed after 24 h of treatment with one or another drug (Fig. 1B, 1C, Supplemental Fig. 1A). At high concentration (≥15 μg/ml), monocytes were susceptible to treatment with both drugs, whereas NK cells were killed by imatinib but virtually resistant to treatment with nilotinib, even at the highest concentration used. At drug concentration ≤6 μg/ml, a low percentage of dead monocytes was detected after treatment with imatinib, whereas with nilotinib high numbers of cells underwent apoptosis and/or necrosis, particularly at 6 μg/ml. NK cells were poorly susceptible to treatment with a low concentration of imatinib and virtually resistant to nilotinib.

To confirm that NK cells were less susceptible than monocytes to the cytotoxic effect of TKIs, cells were treated daily with a drug concentration of ≤6 μg/ml and analyzed for apoptosis/necrosis after 24, 48, and 72 h (Supplemental Fig. 1B). The NB cell line was resistant to treatment with TKIs. The percentage of monocytes undergoing apoptosis/necrosis increased at 48 h, and with drug concentrations ≥3 μg/ml most cells died on day 3. On the contrary, NK cells remained resistant to all nilotinib concentrations even after 3 d of treatment. Two days of treatment with imatinib minimally affected NK cell survival, and relevant numbers of dead cells were detected on day 3 only. This, however, occurred in some donors, whereas in others NK cells were poorly susceptible to imatinib cytotoxicity even after 3 d of treatment.

Imatinib and nilotinib modify the chemokine receptor repertoire of monocytes and NK cells

We analyzed the phenotype of monocytes and NK cells that had been treated with a concentration of ≤6 μg/ml, gating on annexin V and TO-PRO-3 iodide double-negative viable cells. Neither drug altered the expression of a large panel of surface markers, typical of one or another cell type (34, 35) (Supplemental Fig. 1C). On the contrary, imatinib and nilotinib significantly modified the chemokine receptor repertoire of immune cells (Fig. 2A, Supplemental Fig. 1C). In particular, both monocytes and NK cells showed strong upregulation of the expression of CXCR4. Moreover, at 6 μg/ml, downregulation of CCR1 or CXCR3 was detected in monocytes and NK cells, respectively. Neither drug modified the expression of other chemokine receptors, including CX_3CR1, which is expressed by both monocytes and NK cells (Supplemental Fig. 1C).

The drug-mediated upregulation of CXCR4 was observed not only in monocytes and NK cells but also in PB T and B cells

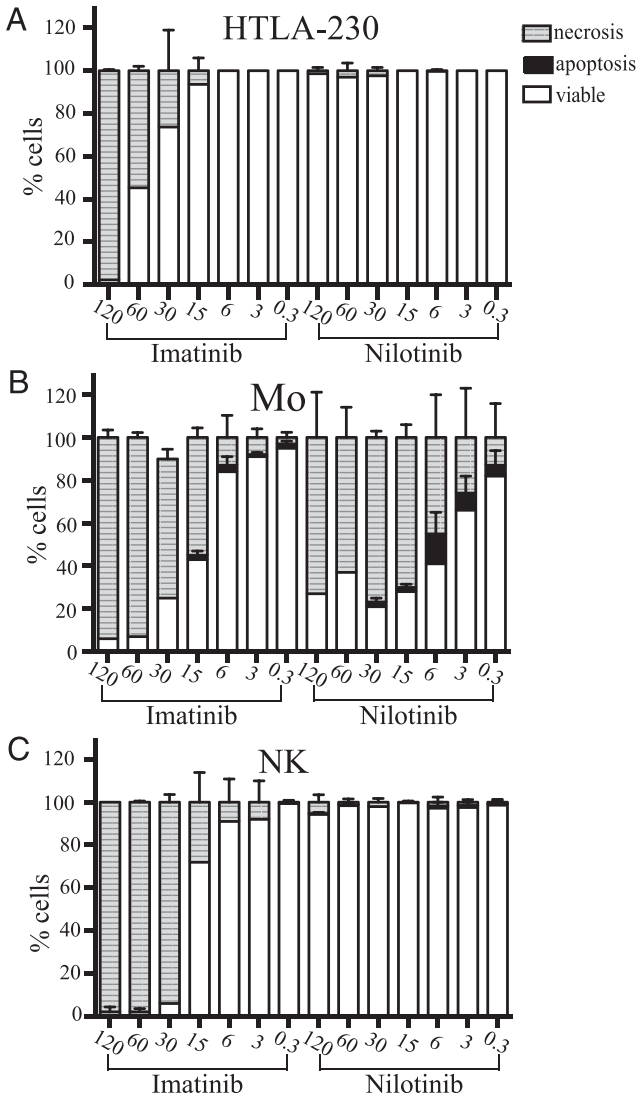

FIGURE 1. Imatinib and nilotinib have different effects on the viability of NB, monocytes, and NK cells. The HTLA-230 NB cell line (**A**), monocytes (Mo) (**B**), and NK cells (**C**) purified from unrelated healthy donors were treated with decreasing concentration of imatinib or nilotinib (micrograms per milliliter) and analyzed by flow cytometry (annexin V and TO-PRO-3 iodide) to identify the percentages of apoptotic, necrotic, or viable cells. Data were normalized considering as 100% the percentage of viable cells in the control (cells cultured in the absence of drugs). Data were pooled from four independent experiments. Mean and 95% confidence intervals are shown.

(Fig. 2B). Notably, neither drug induced significant modulation of CXCR4 expression in NB cell lines that constitutively expressed different levels of the chemokine receptor (Fig. 2B, Supplemental Fig. 1D).

With regard to the mechanism responsible for TKI-mediated upregulation of CXCR4 in immune cells, preliminary results showed regulation at the transcriptional level. Indeed, according to the surface phenotype, imatinib and nilotinib increased CXCR4 mRNA expression in NK cells and monocytes, but not in NB cell lines (Fig. 2C).

Imatinib and nilotinib did not affect or marginally impair cytokine-mediated activation of NK cells

Activated NK cells display cytolytic activity against NB cells, and DNAM-1/PVR interactions play a predominant role in tumor recognition (29). We analyzed whether drug concentrations of ≤6 μg/ml could impair the capability of NK cells to degranulate in the presence of NB. In NB, neither drug modified the expression of ligands for the activating NK receptors DNAM-1 and NKG2D, or induced upregulation of molecules that could limit the NK cell function (Supplemental Fig. 1D). These include HLA class I, B7-H3 (37), and PD-L1 and PD-L2 (38), ligands of the PD-1 inhibitory receptor (39).

Next we analyzed whether TKI conditioning could affect cytokine-mediated activation of NK cells. As shown in Fig. 3, the presence of imatinib and 0.3 μg/ml nilotinib did not significantly impact the acquisition of the CD69 activation marker, and NK cells in the presence of NB showed a degranulation capability comparable to that observed in the absence of TKIs. The only exception was represented by nilotinib used at 6 μg/ml, which hampered degranulation capability, with this effect being paralleled by a reduced upregulation of CD69.

Interestingly, as observed in PB NK cells, all drug concentrations also induced significant upregulation of CXCR4 in cytokine-stimulated NK cells (Fig. 3B).

M0 and M2 macrophages are more resistant to TKIs than monocytes and maintain the capability of polarizing toward M1

We investigated whether imatinib or nilotinib could affect the survival and function of macrophages, in particular of those polarized toward M2 that might display a tumor-promoting role (Fig. 4, Supplemental Fig. 2A–D). Purified PB monocytes were cultured with M-CSF and on day 7 differentiated macrophages (M0) were polarized toward M2 in the presence of IL-4. M0 and M2 macrophages were treated with different concentrations of imatinib or nilotinib and analyzed for apoptosis and/or necrosis by flow cytometry. M0 and M2 were more resistant to TKI exposure (Fig. 4, Supplemental Fig. 2A–D) as compared with monocytes (see Fig. 1B, Supplemental Fig. 1A, 1B). Indeed, imatinib concentrations ≥30 μg/ml were necessary to induce apoptosis/necrosis in a relevant number of macrophages, and cell viability was only marginally affected by TKI concentration up to 15 μg/ml. Moreover, macrophages were highly resistant to all concentrations of nilotinib (Fig. 4, Supplemental Fig. 2A).

The treatment with drug concentrations ≤6 μg/ml did not damage the phagocytosis capability of macrophages, which showed levels comparable to controls (Supplemental Fig. 2B), or their ability to polarize toward M1 via TLR stimulation. Indeed, also in the presence of drugs, LPS stimulation increased the expression of CD80 and HLA class I and abolished that of the membrane-bound form of IL-18 (mIL-18) (40) (Supplemental Fig. 2C). Moreover, after TLR stimulation, M0 and M2 released proinflammatory/immunostimulatory cytokines such as IL-12, TNF-α, and, according to the loss of mIL-18, soluble IL-18 (Supplemental Fig. 2D).

In the presence of imatinib and nilotinib, TLR-stimulated M2 macrophages promote NK cell activation

We analyzed whether imatinib or nilotinib could influence the capability of M1-polarizing macrophages to induce the activation of PB NK cells. Purified PB NK cells were cocultured with autologous M2 macrophages stimulated with LPS, either in the absence or in the presence of TKI concentration ≤6 μg/ml. After 24 h, NK cells were recovered and evaluated for the phenotypic and functional characteristics (Fig. 5, Supplemental Fig. 2E).

In agreement with previously published data (34, 35), TLR-stimulated M2 induced in NK cells upregulation of the expression of CD69, CD25, and CCR7, enhancement of degranulation capability, and the release of high amounts of IFN-γ. In the presence of imatinib, NK cells maintained the capability to upregulate

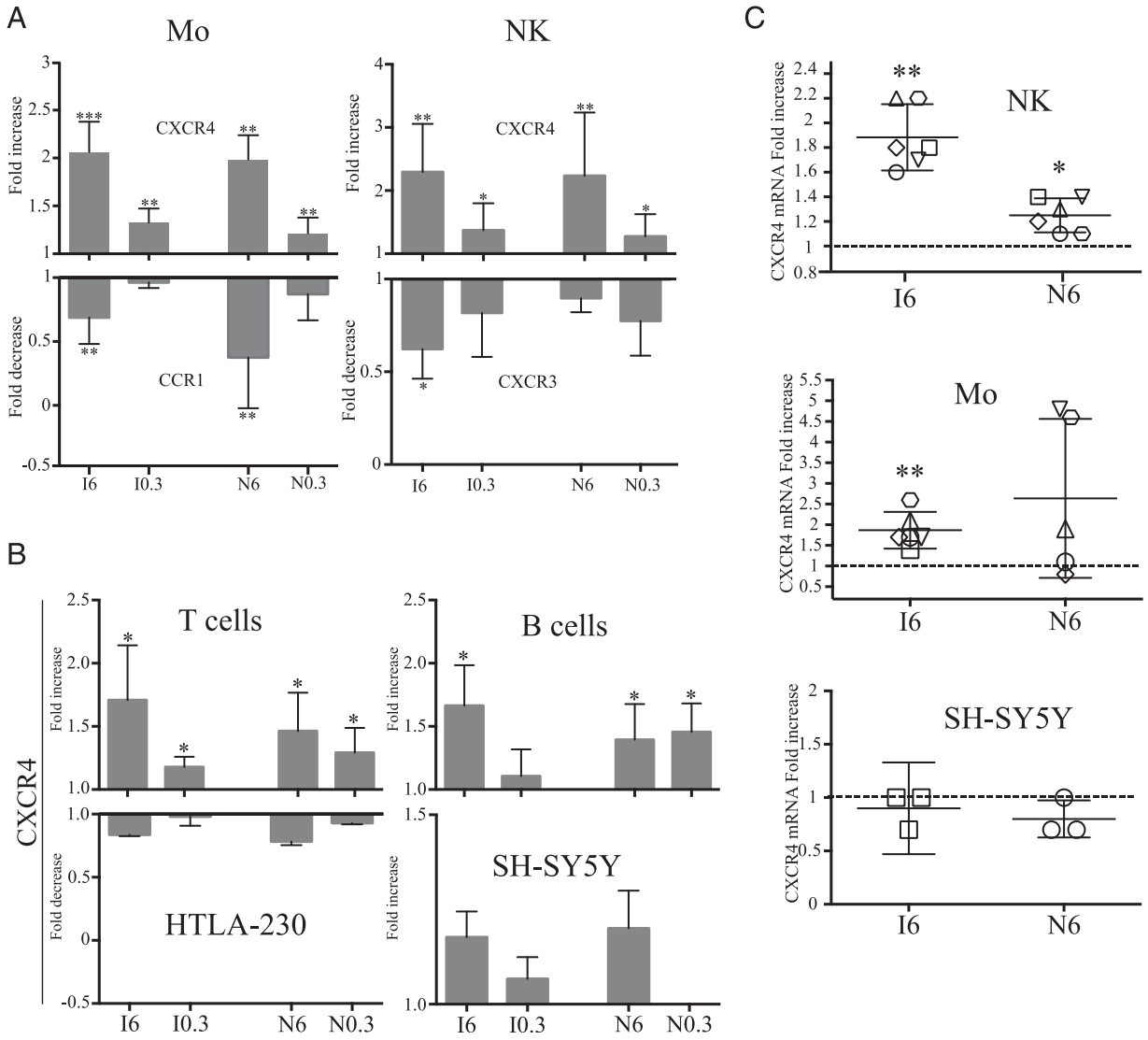

FIGURE 2. Imatinib and nilotinib modify the chemokine receptor repertoire of monocytes and NK cells. (**A**) Purified monocytes (Mo) and NK cells were treated with 6 or 0.3 μg/ml imatinib (I6, I0.3) or nilotinib (N6, N0.3) for 24 h and analyzed by flow cytometry for the expression of the indicated chemokine receptors. (**B**) CXCR4 expression was analyzed by flow cytometry in PBMC T and B cells (gating on $CD3^+$ $CD56^-$ and $CD20^+$ cells, respectively) and in two representative NB cell lines after treatment with I6, I0.3 or N6, N0.3. For (A) and (B), data were pooled from eight independent experiments. (**C**) NK cells and monocytes (five or six unrelated donors) and the SH-SY5Y NB cell line (three independent experiments) were treated with I6 or N6 for 24 h and analyzed by RT-PCR for CXCR4 mRNA expression. Each experiment was performed in triplicate. Fold increase or decrease refers to control represented by untreated cells (arbitrarily normalized to 1). Mean, 95% confidence intervals, and significance ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) are shown.

the CD69 and CD25 expression, to degranulate in the presence of the NB cell line, and released IFN-γ at levels comparable to those observed with the classical rIL-12 plus rIL-18 cytokine stimulation. In the presence of nilotinib at 0.3 μg/ml, NK cells showed CD69 and CD25 upregulation, as well as increased degranulation and IFN-γ production. On the contrary, at a concentration of 6 μg/ml, nilotinib significantly impaired the acquisition of CD69 and the increase of degranulation capability.

In the presence of either TKI, CCR7 expression displayed considerable variability among donors, and only in some cases an increased percentage of $CCR7^+$ cells was detected, particularly when using imatinib.

Imatinib and nilotinib affect the survival of monocytes differentiating toward macrophages in the presence of M-CSF

We also evaluated whether imatinib or nilotinib could affect the capacity of monocytes to differentiate toward macrophages in the presence of M-CSF.

Purified PB monocytes were differentiated toward M0 with M-CSF in the presence of TKIs. Cells were analyzed by flow cytometry for cell survival rate and, gating on viable cells, for the expression of surface markers typical of macrophages (Fig. 6A, Supplemental Fig. 3A, 3B). At drug concentration of 6 μg/ml most cells underwent apoptosis/necrosis. At 0.3 μg/ml, high percentages of viable cells were detected, which progressively acquired a phenotype typical of macrophages. In particular, cells reduced the expression of CD14 and HLA II, increased that of CD16, and de novo expressed NTB-A, B7-H3, CD204, and mIL-18 (Supplemental Fig. 3B) (34, 40).

We evaluated whether the reduced numbers of surviving and differentiating monocytes could correlate with a drug-mediated reduction of the expression of the M-CSF receptor (CSF1R) (Fig. 6B, Supplemental Fig. 3C). Purified PB monocytes were treated with imatinib and nilotinib for 24 h and analyzed by flow cytometry. Exposure to TKI concentrations of 6 μg/ml strongly decreased both the percentage of monocytes expressing M-CSFR

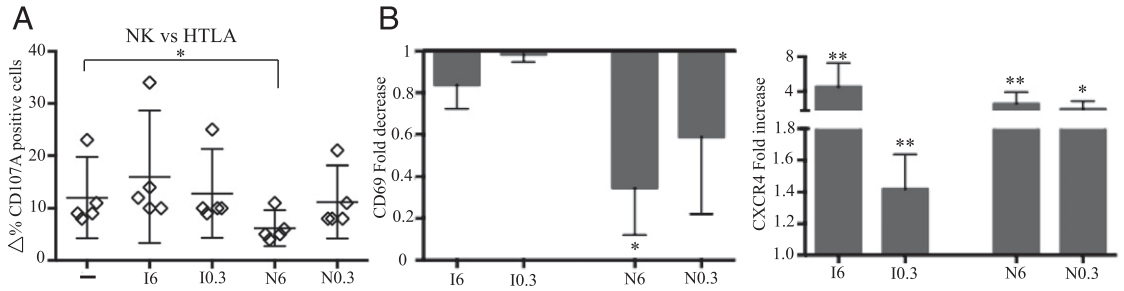

FIGURE 3. Imatinib and nilotinib marginally affect the cytokine-dependent activation of NK cells. (**A**) Purified PB NK cells were stimulated with rIL-12 and rIL-18 either in the absence (−) or in the presence of I6, I0.3 or N6, N0.3 and analyzed by flow cytometry for degranulation (CD107a assay) in the presence of HTLA-230. The change in percentage (Δ%) refers to the percentage of $CD107a^+$ NK cells with target minus the percentage of $CD107a^+$ NK cells without target. (**B**) NK cells treated as in (A) were analyzed for the expression of CD69 and CXCR4. Fold decrease or increase versus control (cytokine-stimulated NK cells without drugs, arbitrarily normalized to 1) is shown. Data pooled from five independent experiments. Mean, 95% confidence intervals, and significance ($*p < 0.05$, $**p < 0.01$) are shown.

and its surface density. On the contrary, treatment with 0.3 μg/ml did not significantly affect M-CSFR expression.

Effect of imatinib on immune cells from high-risk NB and CML patients

PBMCs were obtained from seven children with high-risk NB either at the disease onset or relapsing and analyzed for CXCR4 expression. In these patients, NK cells and monocytes constitutively expressed levels of the chemokine receptor comparable to those of healthy donors. In vitro treatment with imatinib (6 μg/ml) induced upregulation of CXCR4 expression in both cell types (Fig. 7A, Supplemental Fig. 4A).

We extended the analysis to PBMCs from three adult and two pediatric patients receiving imatinib for CML. CML NK cells and monocytes displayed a higher CXCR4 surface expression as compared with healthy donors (Fig. 7B, Supplemental Fig. 4B). The chemokine receptor surface density further increased when CML PBMCs were treated in vitro with imatinib at the concentration of 6 μg/ml (Fig. 7B, Supplemental Fig. 4B). Moreover, the treatment downregulated the expression of CCR1 and M-CSFR in monocytes (Fig. 7C, Supplemental Fig. 4B). Unlike what was observed in healthy donors (see Fig. 2A, Supplemental Fig. 1C), in vitro imatinib exposure did not decrease the expression of CXCR3 in CML NK cells (Supplemental Fig. 4B).

NK cells of CML patients were also analyzed for degranulation in the presence of the prototypical K562 target cell line (Fig. 7D, Supplemental Fig. 4C). The degranulation capability of resting NK cells did not significantly differ from that of healthy donors. Moreover, the in vitro treatment with imatinib did not affect the degranulation capability of resting and cytokine-stimulated CML NK cells. Notably, in some patients NK cells appeared to degranulate better in the presence than in the absence of the drug.

Discussion

More than 20 TKIs are approved as cancer therapies for patients with Ph+ hematological malignancies, melanoma, breast cancer, non–small cell lung cancer, and colorectal cancer. Recently it has been demonstrated that imatinib provides clinical benefits in GISTs (18) and NB (15). In particular, in GISTs, benefits were associated with drug-dependent off-target effects, such as modulation of immune responses, and in NB a similar mechanism was postulated to occur. This prompted us to analyze the effect of two TKIs, namely imatinib and nilotinib, on NK cells, which represent pivotal cytolytic effectors in antitumor responses. Moreover, owing to the existence of a crosstalk between NK cells and macrophages (34), the analysis was extended to monocytes and macrophages, with particular attention to those characterized by tumor-promoting M2 polarization. TKI concentrations ≥15 μg/ml are higher than those achievable in adult patients in which plasma concentrations between 1 and 3 μg/ml are presently recommended (1). In pediatric patients, who showed higher tolerability than adults, plasma concentrations of imatinib ranged from 2 to 7 μg/ml, with lower values associated with better responses (15). We thus selected drug concentrations ≤6 μg/ml that, under our experimental conditions, were devoid of direct effects on NB cells and we analyzed their influence on phenotype and function of immune cells.

PB (resting) NK cells were highly resistant to the cytotoxic effect of the drugs, as <10% of cells died when exposed to imatinib and virtually all survived when exposed to nilotinib. Moreover, with the exception of nilotinib used at 6 μg/ml, NK cells maintained

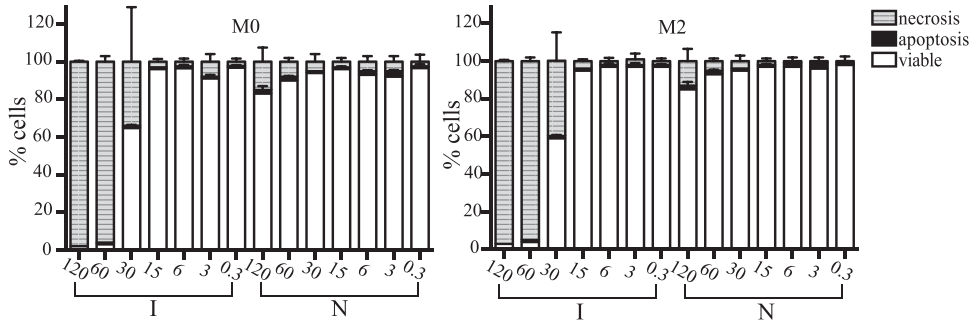

FIGURE 4. Macrophages are highly resistant to imatinib and nilotinib. M0 and M2 cells were treated with the indicated concentrations (micrograms per milliliter) of imatinib or nilotinib and analyzed for flow cytometry (annexin V and TO-PRO-3 iodide) to identify the percentage of necrotic, apoptotic, and viable cells. Data were normalized considering as 100% the percentage of viable cells in the control (untreated macrophages). Data were pooled from three independent experiments. Mean and 95% confidence intervals are shown.

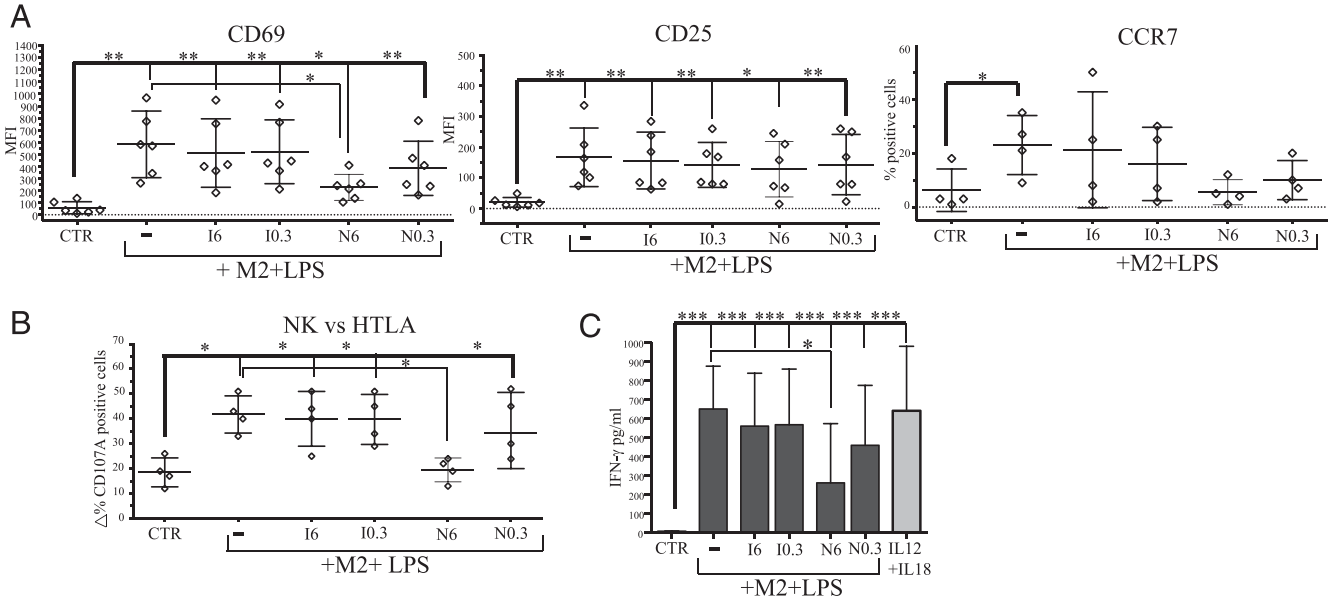

FIGURE 5. Imatinib and nilotinib do not significantly affect the capability of M1-polarizing macrophages to activate PB NK cells. Purified PB NK cells were cultured alone (CTR) or with autologous M2 stimulated with LPS either in the absence (−) or in the presence of I6, I0.3 or N6, N0.3. NK cells were recovered and analyzed by flow cytometry for (**A**) the surface expression of CD69, CD25, and CCR7 and (**B**) degranulation capability in the presence of HTLA-230 cell line (CD107a assay). The change in percentage (Δ%) refers to the percentage of $CD107a^+$ NK cells with target minus the percentage of $CD107a^+$ NK cells without target. (**C**) IFN-γ release (culture supernatants, ELISA assay). IFN-γ release by NK cells stimulated with rIL-12 plus rIL-18 is shown as positive control. Data were pooled from six (A), four (B), and eight (C) independent experiments. Mean, 95% confidence intervals, and significance ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) are shown.

the ability to be activated by immunostimulatory cytokines, increasing the expression of activation markers and acquiring a degranulation capability in the presence of NB cells, comparable to that detected in the absence of drugs. In this context, in NB cells neither drug substantially modified the expression of ligands for the activating NK receptors (14) or induced upregulation of HLA class I and of other molecules involved in the immune checkpoints (38, 41, 42). Importantly, the drugs did not substantially alter the expression in NK cells of activating receptors, including DNAM-1 and NKp30, which are crucial for recognition and killing of both leukemia (23) and NB cells (29). Of note, a certain degree of variability in NKp30 surface expression upon TKI exposure was detected among donors. This could be explained by changes in the expression levels of the NKp30 receptor isoforms (a, b, and c)

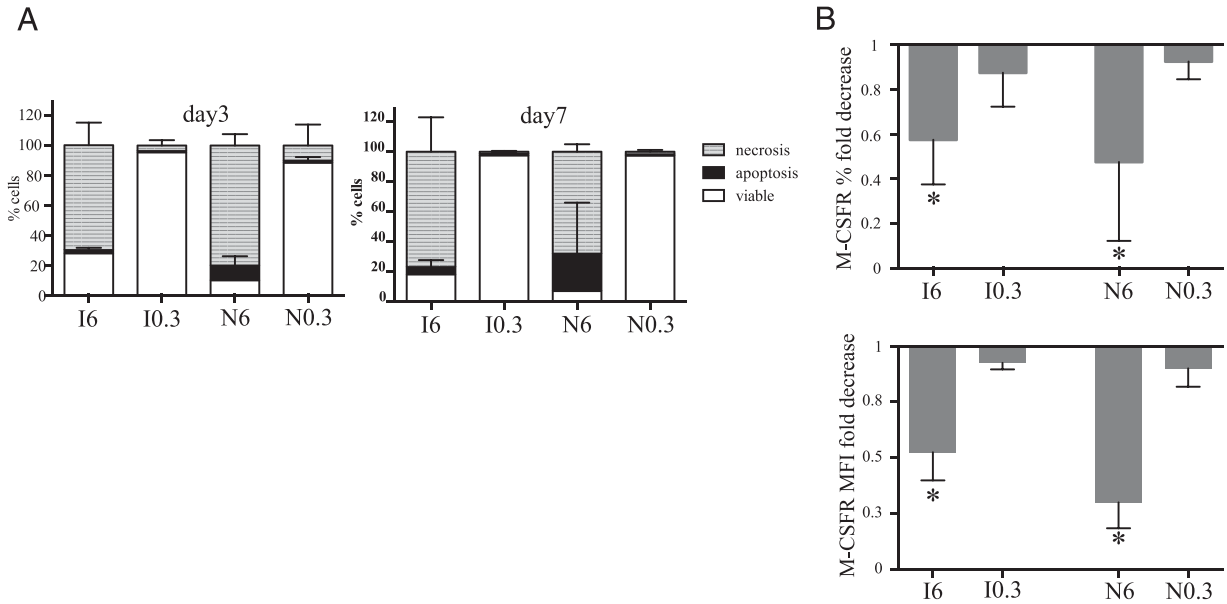

FIGURE 6. Imatinib and nilotinib reduce the number of monocytes differentiating toward macrophages. (**A**) Purified PB monocytes were cultured in the presence of M-CSF either in the absence or in the presence of I6, I0.3 or N6, N0.3 and analyzed (on days 3 and 7) by flow cytometry (annexin V and TO-PRO-3 iodide) to identify the percentage of necrotic, apoptotic, and viable cells. Data were normalized considering as 100% the percentage of viable cells in the control (monocytes with M-CSF, without drugs). (**B**) PB monocytes were treated with I6, I0.3 or N6, N0.3 for 24 h and, gating on viable cells, analyzed by flow cytometry for M-CSFR expression. The percentage or mean fluorescence intensity (MFI) fold decrease versus control (untreated monocytes, arbitrarily normalized to 1) is shown. Data were pooled from eight independent experiments. Mean, 95% confidence intervals, and significance ($*p < 0.05$) are shown.

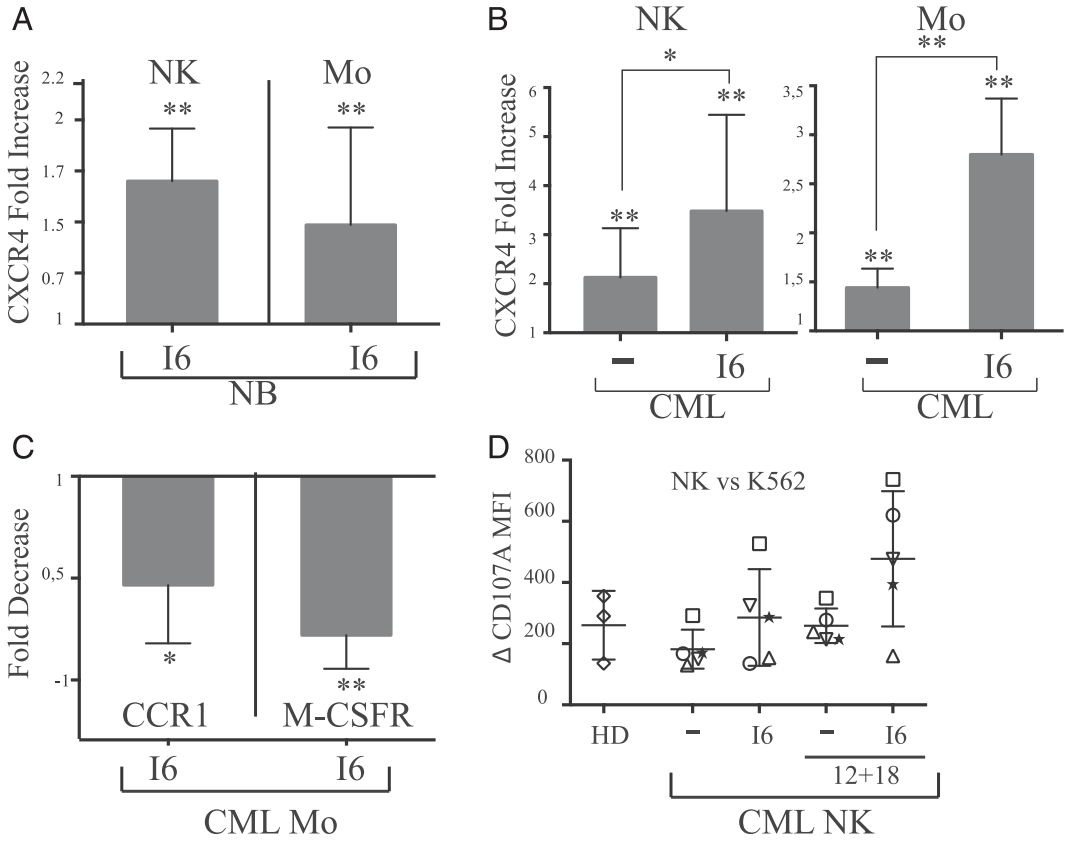

FIGURE 7. Imatinib effects in NK and monocytes from NB and CML patients. (**A**) PBMCs from seven NB patients were treated in vitro with I6 for 24 h and analyzed by flow cytometry for CXCR4 expression, gating on NK cells ($CD3^-CD56^+$) or monocytes (Mo; $CD14^+$). Fold increase versus control (untreated NB PBMCs, arbitrarily normalized to 1) is shown. (**B**) PBMCs from five CML patients either untreated (−) or treated in vitro with I6 for 24 h were analyzed by flow cytometry for CXCR4 expression gating on NK cells or monocytes. Fold increase versus controls (NK cells or monocytes from three untreated healthy donors, arbitrary normalized to 1) is shown. (**C**) CML PBMCs were treated in vitro with I6 for 24 h and analyzed by flow cytometry for CCR1 and M-CSFR expression, gating on monocytes. Fold decrease versus control (untreated CML PBMCs, arbitrarily normalized to 1) is shown. (**D**) PBMCs of five CML patients, untreated (−) or I6 treated, were analyzed for NK cell degranulation (CD107a assay) gating on NK cells, either in the absence or in the presence of stimulatory cytokines (rIL-12 plus rIL-18). PBMCs of three healthy donors (HD) are shown as controls. The change in mean fluorescence intensity (ΔMFI) refers to MFI of $CD107a^+$ NK cells with target (K562) minus MFI of $CD107a^+$ NK cells without target. Mean, 95% confidence intervals, and significance ($*p < 0.05$, $**p < 0.01$) are shown.

(43). This interpretive hypothesis is under further investigation, because it could correlate with differences in responses to drug treatments. Indeed, recent data showed a reduced response to imatinib in GIST patients with the NKp30 isoform c, which induces production of IL-10 instead of IFN-γ and TNF-α (18).

Importantly, both TKIs revealed a striking capacity to modulate the chemokine receptor repertoire of NK cells. In particular, they strongly increased CXCR4 expression in both resting and activated NK cells, and imatinib at the highest concentration used decreased that of CXCR3. Interestingly, increased CXCR4 expression upon TKI exposure was detected also in PB T and B lymphocytes, but it did not occur in NB cells. These data suggest that imatinib and nilotinib through CXCR3 downregulation and CXCR4 upregulation might disfavor the recruitment of NK cells toward peripheral tissues, while supporting their homing into BM niches. This might potentiate the NK immune surveillance against tumor cells that, as occur in stage 4 NB patients, metastasize in BM. It is of note that, different from what has been described for TGF-β (33), imatinib and nilotinib did not modify the expression of CX_3CR1, which participates in the recruitment and extravasation of NK cells into BM.

Increased CXCR4 expression upon TKI exposure also occurred in PB monocytes and was accompanied by downregulation of CCR1, which participates in their extravasation in peripheral tissues. Monocytes were more susceptible than NK cells to drug exposure and, in particular, at 6 μg/ml, significant numbers of cells underwent apoptosis/necrosis with imatinib and up to 60% died with nilotinib. This effect was not prevented by stimulation with M-CSF, the major factor involved in monocyte differentiation toward macrophages (44). In keeping with the low survival rate and the reduced number of cells undergoing differentiation, in the presence of TKIs, monocytes strongly decreased the expression of the M-CSFR. These results are in line with previous data showing that M-CSF is fundamental for survival of monocytes (45, 46) and that imatinib impairs the signal transduction pathway of the M-CSFR (47). Our data suggest that imatinib and nilotinib could decrease in vivo the half-life of circulating monocytes, as well as their recruitment and macrophage differentiation in tissues, possibly reducing the generation of macrophages that in the tumor microenvironment might acquire M2 tumor-promoting function. Interestingly, it has been shown that imatinib inhibited the early phase of the differentiation of myeloid-derived suppressor cells and reduced their number in CML patients (48), whereas it did not affect differentiation toward dendritic cells (49).

Macrophages, both unpolarized (M0) and M2, were highly resistant to imatinib and nilotinib. A high number of cells survived to TKI exposure and maintained their phenotypic and functional characteristics, thus showing that drugs did not induce or modify macrophage polarization. Importantly, in the presence of TKIs, M2

maintained the capability to revert their immunosuppressive functional phenotype toward M1 after TLR engagement. Indeed, they released high amounts of proinflammatory and immunostimulatory cytokines. Moreover, with the exception of nilotinib used at 6 μg/ml, M1-polarized M2 macrophages acquired the capability of fully activating NK cells that increased the expression of the α-chain of the IL-2 receptor, the degranulation capability in the presence of NB, and released a high amount of IFN-γ, a cytokine crucial for amplification of both innate and adaptive Th1 antitumor immune responses.

Collectively, our results shed light on the influence of imatinib mesylate and nilotinib on NK cells and monocyte/macrophage survival, function, and expression of chemokine receptors, contributing both to better interpreting the efficacy of these agents in tumors and to envisaging strategies aimed at facilitating an antitumor immune response rather than to promoting a direct effect on tumor cells.

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Disclosures

A.M. is a founder and shareholder of Innate Pharma (Marseille, France). The remaining authors have no financial conflicts of interest.

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Research Paper

NK cells and multiple myeloma-associated endothelial cells: molecular interactions and influence of IL-27

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ABSTRACT

Angiogenesis represents a hallmark of tumor progression in Multiple Myeloma (MM), a still incurable malignancy. Here we analyzed the activity of cytokine-stimulated NK cells against tumor-associated endothelial cells isolated from bone marrow aspirates of MM patients with active disease (MMECs). We show that NK cells activated with optimal doses of IL-15 killed MMECs thanks to the concerted action of multiple activating receptors. In particular, according to the high expression of PVR and Nectin-2 on MMECs, DNAM-1 actively participated in target recognition. Interestingly, in MMECs the surface density of PVR was significantly higher than that detected in endothelium from patients with MM in complete remission or with monoclonal gammopathy of undetermined significance (MGUS). Importantly, IL-27, which unlike IL-15 does not display pro-angiogenic properties, maintained or increased the NK cell functions induced by suboptimal concentrations of IL-15. NK cell properties included killing of MMECs, IFN-γ production as well as a peculiar increase of NKp46 expression on NK cell surface. Finally, IL-27 showed a striking capability of up-regulating the expression of PD-L2 and HLA-I on tumor endothelium, whereas it did not modify that of PD-L1 and HLA-II.

Our results suggest that cytokine-activated endogenous or adoptively transferred NK cells might support conventional therapies improving the outcome of MM patients.

INTRODUCTION

In the last decades important advancements in the treatment of different malignances have led to significant improvement in patients' survival. A variety of therapies are currently available including those targeting specific pathways involved in the growth and survival of cancer cells or tumor-associated stromal cells such as endothelial cells. A pro-tumoral crosstalk between cancer and endothelial cells occurs and is essential for tumor growth. In multiple myeloma (MM), an incurable malignancy of monoclonal plasma cells [1], angiogenesis represents a hallmark of tumor progression and anti-VEGF drugs, alone or in combination with other agents, are currently used. In some patients however, available therapeutic approaches resulted in limited benefits and in a short-lasting tumor regression. Thus, many efforts have been made to evaluate additional therapeutic protocols aimed at obtaining a more durable tumor control in different malignances including MM. New strategies consist of immunotherapeutic approaches including the strengthening of the function of endogenous Natural

Killer (NK) cells or the adoptive transfer of "armed" or activated NK cells [2–5]. These cells are cytotoxic members of the Innate Lymphoid Cell (ILCs) family [6, 7] and have been shown to play a crucial role in tumor surveillance also due to their capability of killing tumor cells including those with stem cell like properties [8–11]. The mechanisms allowing the NK-mediated recognition of tumor cells have been largely clarified and consist of the cooperation of different triggering receptors that are engaged by specific ligands upregulated or de novo expressed on transformed cells [3, 11, 12]. Activating NK receptors are represented by NKp46 (CD335), NKp30 (CD337) and NKp44 (CD336) (collectively termed natural cytotoxicity receptors, NCR), DNAM-1 (CD226) and NKG2D (CD314) [3, 13]. Although viral glycoproteins have been identified as ligands for NCR, their cellular ligands are not fully defined [14]. Two molecules, the mixed-lineage leukemia (MLL5) [15] and B7-H6 [16] have been found to be expressed on a wide panel of tumors [17] and represent cellular surface ligands of NKp44 and NKp30, respectively. It is of note however, that different evidences suggest that NKp30 would be able to recognize additional, still undefined, tumor-associated ligand(s). NKG2D recognizes MICA/B and ULBPs [12] stress inducible molecules de-novo expressed after tumor transformation and virus infection. DNAM-1 binds PVR (CD155) and Nectin-2 [18], two members of the Nectin family that are also recognized by Tactile [19] and TIGIT [20]. PVR and Nectin-2 are over-expressed in tumors of different histotype and their interaction with DNAM-1 is non-redundant and crucial to obtain an efficient tumor cell killing [21–24]. Accordingly, different studies highlighted the importance of DNAM-1/ligands interactions in the establishment of the activating immunological synapse that allows tumor recognition by both NK and T cells [25–28].

The possible effect of NK-cell based therapeutic approaches in killing of tumor-associated endothelial cells with the consequent reduction of the vascular network remains to be determined.

In the present study we analyzed the NK cell activity against tumor-associated endothelial cells (EC) isolated from bone marrow aspirates of MM patients in active phase (MMECs). We evaluated the susceptibility of MMECs to killing mediated by IL-15-stimulated NK cells and dissected the molecular interactions occurring between effector and target cells. Moreover, we investigated the immunostimulatory effects of IL-27 [29] that, unlike IL-15, does not display pro-angiogenic properties [30, 31]. Finally, considering that NK cell activation might result *in vivo* in a cytokine storm responsible for the activation of immune checkpoints, [32, 33] we analyzed in MMECs the constitutive and cytokine-induced surface expression of Programmed Death Ligands (PD-Ls) and HLA class I and II [34–36].

RESULTS

DNAM-1 actively participates to the killing of MMECs mediated by rIL-15-activated NK cells

Tumor-associated endothelial cells were isolated from bone marrow (BM) aspirates of nine Multiple Myeloma Patients in active phase (Table 1) [37]. MMECs were analyzed for the susceptibility to lysis mediated by peripheral blood mononuclear cells (PBMCs) of healthy donors activated with optimal doses of rIL-15 (20 ng/ml) (Figure 1A). Overall, activated PBMCs killed the MMECs analyzed and HLA class I molecules had a poor protective role as demonstrated by the lack of significant differences observed in the presence of the anti-HLA-I mAb (Figure 1A). It is of note however that a certain degree of heterogeneity in the susceptibility of MMECs to activated PMBCs could be appreciated. Indeed, MMEC3 and MMEC4 showed a susceptibility to lysis comparable to that of EA, a prototypic tumor endothelial cell line used as control, whereas MMEC1 and MMEC2 were more resistant (Figure 1A).

Assuming a predominant role of NK lymphocytes in the killing of MMECs by rIL-15 activated PBMCs, we analyzed the susceptibility of MMECs to lysis mediated by highly purified activated NK cells (Figure 1B). Moreover, in order to analyze the possible contribution of the different activating NK receptors in the recognition of MMECs, cytolytic assays were performed in the presence of mAbs able to specifically disrupt the interactions between the receptors (on NK cells) and their ligands (on target cells). Similar to EA, MMECs were highly susceptible to killing mediated by rIL-15 activated NK cells, a process that depended on the cooperation of various activating receptors (Figure 1B). In particular, NKG2D and DNAM-1 contributed to the killing of MMEC3 and a significant inhibition of lysis was observed only after the combined mAb-mediated masking of both molecules. NKG2D was not involved in MMEC5 recognition, whereas DNAM-1 played a major role in the NK-mediated cytotoxicity, as its mAb-mediated masking resulted in a significant reduction of lysis. Moreover, mAb-mediated masking of NKp30 and NKp46 significantly reduced the lysis demonstrating the involvement of these receptors in killing of MMEC5 (Figure 1B). The NK-mediated recognition of EA cells involved the four different activating receptors thus recapitulating what observed in endothelial cells derived from MM patients. A similar scenario was observed using endothelial cells obtained from patients with monoclonal gammopathy of undetermined significance (MGECs). In these experiments we used the CD107a assay that was more suitable to preserve the viability of target cells. As shown in Supplementary Figure 1, rIL-15 stimulated NK cells degranulated in the presence of MGECs (and in the presence of EA, used as control) and DNAM-1, NKG2D, NKp30 and NKp46 receptors clearly cooperated in the process.

Table 1: Endothelial cells analyzed in the study

Endothelial cells	Disease	Phase	Age	Gender
MMEC1	MM	Active	55	Male
MMEC2	MM	Active	63	Female
MMEC3	MM	Active	70	Male
MMEC4	MM	Active	67	Female
MMEC5	MM	Active	73	Female
MMEC6	MM	Active	55	Male
MMEC7	MM	Active	65	Male
MMEC8	MM	Active	60	Female
MMEC9	MM	Active	63	Female
cr-MMEC	MM	Complete remission	71	Male
MGEC1	MGUS		47	Male
MGEC2	MGUS		59	Male
MGEC3	MGUS		61	Female
MGEC4	MGUS		75	Female
MGEC5	MGUS		53	Male
MGEC6	MGUS		75	Male
MGEC7	MGUS		64	Male
IDAEC	IDA		56	Female

The endothelial cells analyzed in this study were derived from bone marrow aspirates of patients with Multiple Myeloma in active phase (MMEC1-9) or complete remission (cr-MMEC), patients with monoclonal gammopathy of undetermined significance (MGEC1-7) and a patient with anemia due to iron deficiency (IDAEC).

MMECs and EA cell line express the ligands of DNAM-1 activating receptor

MMECs were analyzed for the surface expression of the ligands of activating receptors known to regulate NK cell functions including cytolytic activity. The gating strategy is shown in Supplementary Figure 2. For comparison, the analysis was performed on endothelial cells derived from BM of patients with MM in complete remission (cr-MMEC), monoclonal gammopathy of undetermined significance (MGEC 1-5) or anemia due to iron deficiency (IDAEC).

In all cells analyzed NKG2D-ligands were either undetectable or expressed at very low levels (Table 2). In particular, according to the involvement of NKG2D in NK-mediated lysis (see Figure 1B) MMEC3 expressed MICA, ULBP-2 and ULBP-3. The latter two ligands were also detected in the EA cell line whereas MMEC5, which was killed in an NKG2D-independent manner, did not express any of the NKG2D ligands analyzed. All endothelial cells analyzed expressed good levels of PVR and Nectin-2 (Table 2), ligands of DNAM-1 receptor involved in the killing of MMECs and EA (see Figure 1B). Interestingly, in MMECs and EA the surface density of PVR was significantly higher than in cr-MMEC, MGECs and IDAEC (Figure 2A). Accordingly, MMECs and EA but not the other endothelial cells analyzed were stained by the DNAM-1-Fc soluble receptor (Figure 2B).

In spite of the clear involvement of NKp30 in the NK-mediated lysis of some MMECs and EA cells, none of the endothelial cells analyzed was stained by the NKp30-Fc soluble receptor (Figure 2B), which efficiently bound the B7-H6 specific ligand on B7-H6+ cell transfectants and K562 cell line (Supplementary Figure 3A). EA cell line was characterized by a very low level of B7-H6 transcript, comparable to that detected in immature Dendritic cells (iDC), whose interaction with NK cells is mostly NKp30-dependent [38, 39] (Supplementary Figure 3B). Lack of staining of NKp30-Fc soluble receptor on MMECs might be due to the poor surface expression of B7-H6, not sufficient to bind NKp30-Fc soluble receptor, but capable of engaging the native NKp30 receptor. Alternatively, both MMECs and iDC may express an additional, not yet identified, NKp30 ligand that is not recognized by the NKp30-Fc soluble receptor.

rIL-27 plus suboptimal doses of rIL-15 enhance NK cell cytotoxicity against tumor endothelial cells

NK cells stimulated with optimal doses of rIL-15 displayed cytotoxicity against all MM-associated endothelial cells analyzed, an effect that was accompanied by the upregulated expression of CD69 activation marker (Figure 3B and 3C), NKG2D (Figure 3D) and NKp30 (Figure 3E) and by a modest but significant production of IFN-γ (Figure 3H). However, IL-15 might exert

unwanted *in vivo* side effects due to its strong pro-angiogenic activity. Thus, we analyzed the function of NK cells cultured in the presence of rIL-27 (100 ng/ml), which has been described to combine immunostimulatory and anti-angiogenic properties. As shown in Figure 3, the stimulatory capability of rIL-27 was lower than that of rIL-15. In particular, rIL-27 induced a slight increase in NK cell-mediated killing of EA cells (Figure 3A), a modest increase in the surface expression of CD69 and NKG2D, and did not stimulate the release of IFN-γ. It is of note, however, that rIL-27 caused a peculiar upregulation of the NKp46 receptor (Figure 3F). When used in combination with rIL-15, rIL-27 did not improve CD69 and NKG2D upregulation but significantly increased the cytolytic activity, the surface expression of NKp30 and the IFN-γ production as compared to rIL-15 alone (Figure 3). DNAM-1 expression was not modified by rIL-27 or rIL-15 used either alone or in combination (Figure 3G).

To determine the dose of cytokines suitable to induce NK cell activation minimizing pro-angiogenic side effects, NK cells were treated with rIL-27 in combination with decreasing concentration of rIL-15, ranging from 20 to 1 ng/ml (Figure 4). NK cells stimulated with rIL-27 and the lowest rIL-15 concentration used (1 ng/ml) showed a significant increased expression of CD69 (in terms of both % of positive cells and MFI) and levels of degranulation in the presence of EA cells that reached those observed in the presence of optimal doses of rIL-15. Moreover, rIL-27 promoted an upregulation of DNAM-1 expression (Figure 4C) that progressively increased by reducing the concentration of rIL-15. The ability of rIL-27 to increase NK cell degranulation in the presence of suboptimal doses of rIL-15 (1 ng/ml) was confirmed using as targets both MMECs and MGECs (Figure 5).

rIL-27 up-regulates PD-L2 and HLA class I expression in tumor endothelial cells

We analyzed whether the positive effects of the rIL-$15^{1\ ng}$-rIL-$27^{100\ ng}$ combination on NK cell functions could be associated with side effects such as the up-regulation of ligands capable to inhibit the NK cell-mediated attack.

The EA cell line was analyzed for the expression of the PD-Ls and HLA-II immune-checkpoints molecules,

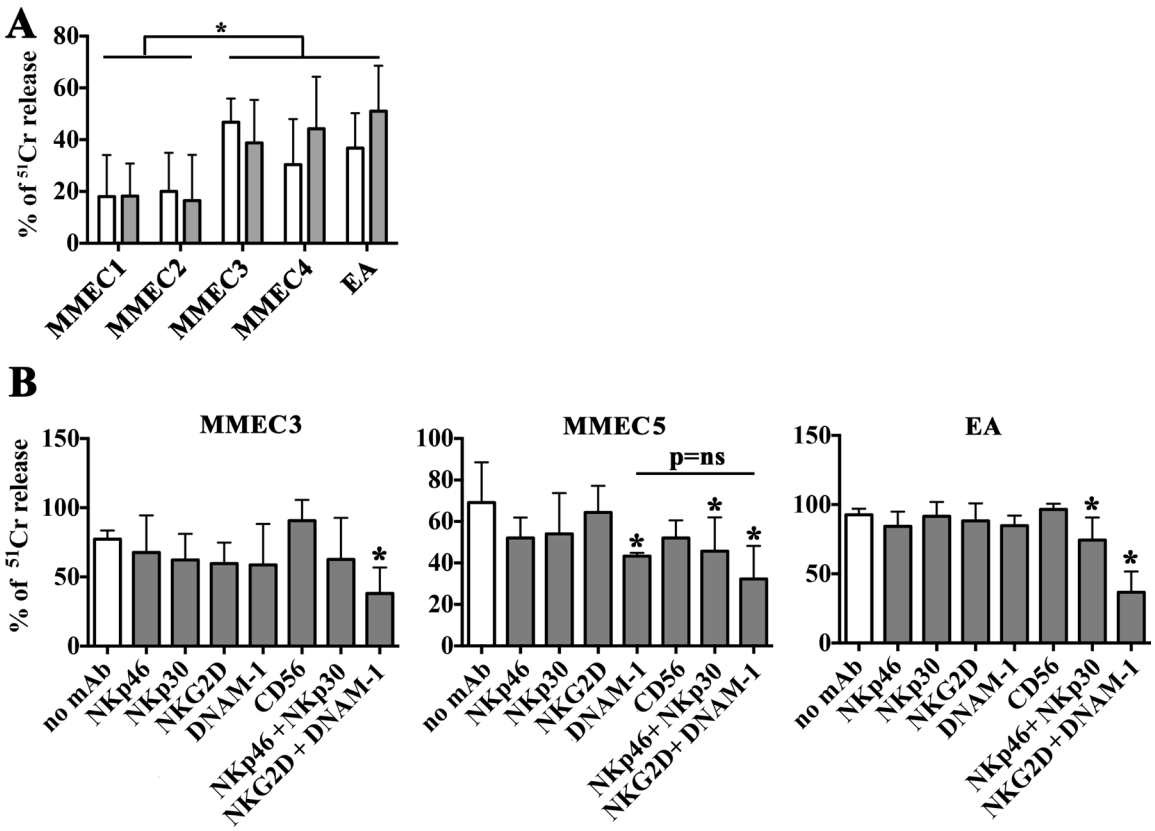

Figure 1: Susceptibility of MMECs to NK-mediated killing and activating receptors involved. (**A**) PBMCs from 3 healthy donors were stimulated with rIL-15 (20 ng/ml) and analyzed for their cytolytic activity (^{51}Cr release assay) against MMECs derived from 4 different patients (MMEC1-4), (E:T ratio 80:1) in the absence (white bars) or in the presence of anti-HLA-I mAbs (gray bars). Mean (3 healthy donors in duplicate), 95% confidence intervals and significance are indicated. *$p < 0.05$. (**B**) IL-15 activated NK cell populations were analyzed for their cytolytic activity (^{51}Cr release assay) against MMECs and EA cell line (E:T ratio 20:1) in the absence (white bars) or in the presence of mAbs (10 μg/ml) specific for the indicated activating NK receptors used alone or in combination. Mean (3 healthy donors in duplicate), 95% confidence intervals and significance are indicated. *$p < 0.05$.

Table 2: NKG2D and DNAM-1 ligands expression in endothelial cells

	ULBP-1	ULBP-2	ULBP-3	ULBP-4	MICA	PVR	Nectin-2
MMEC1	(−)	10	11	(−)	(−)	186	40
MMEC2	(−)	ND	ND	ND	ND	147	43
MMEC3	(−)	7	13	(−)	12	93	44
MMEC4	(−)	(−)	10	(−)	ND	93	24
MMEC5	(−)	(−)	(−)	(−)	(−)	65	23
cr-MMEC	(−)	(−)	(−)	(−)	(−)	38	36
MGEC1	(−)	12	(−)	(−)	(−)	57	24
MGEC2	ND	(−)	9	ND	ND	35	21
MGEC3	(−)	10	10	13	(−)	49	19
MGEC4	(−)	(−)	(−)	6	(−)	61	40
MGEC5	(−)	6	(−)	ND	ND	57	13
IDAEC	ND	(−)	(−)	ND	ND	10	14
EA	(−)	12	24	(−)	(−)	257	57

Endothelial cells used in this study were analyzed by flow cytometry for the expression of the indicated ligands. Values indicate the MFI. Undetectable = (–), ND = not determined. For EA cell line values represent the mean of 10 different experiments.

as well as of HLA-I. As shown in Figure 6, EA cells constitutively expressed PD-L1, PD-L2 and HLA-I. Similar to what has been demonstrated in tumor cells [34, 40], rIFN-γ up-regulated the PD-Ls and HLA-I expression and de novo induced that of HLA-II, whereas rTNFα was effective only in the induction of HLA-I expression.

EA cells were treated with culture supernatants derived from NK cells stimulated with the rIL-$15^{1\ ng}$-rIL-$27^{100\ ng}$ combination. EA cells did not modify the expression of PD-L1 and HLA-II, whereas they showed upregulation of PD-L2 and HLA-I (Figure 6) at levels comparable to those obtained using rIFN-γ. It is of note that a similar effect was obtained using supernatants from NK cells stimulated with rIL-$27^{100\ ng}$ but not with rIL-$15^{1\ ng}$ (Figure 6). Moreover, negligible amounts of IFN-γ were detected in the supernatants of NK cells stimulated with rIL-$15^{1\ ng}$ and rIL-$27^{100\ ng}$, both used alone or in combination (Supplementary Table 1). Overall the data suggested that the increased expression of PD-L2 and HLA-I in EA cells treated with NK supernatants was mainly rIL-27 dependent and IFN-γ/IL-15 independent. To confirm this hypothesis, EA cells were directly stimulated with the cytokines either alone or in combination (Figure 7 and Supplementary Figure 4). rIL-27 alone did not modify the expression of PD-L1 and HLA-II, whereas increased that of PD-L2 and HLA-I that reached levels comparable to those obtained using the rIL-15^{1ng}-rIL-$27^{100\ ng}$ combination. On the contrary, rIL-15 alone had no effect even at the highest (20 ng/ml) concentration used.

The rIL-27 capability of up-regulating HLA-I and PD-L2 molecules in tumor endothelium was confirmed using three different MMECs that were treated with rIL-$27^{100\ ng}$ (or IFN-γ, as control) (Figure 8). According to results obtained on EA, rIL-27 did not significantly modify the PD-L1 expression whereas it up-regulated HLA-I expression in all MMECs, although at different extent. Increased PD-L2 expression was detected in MMEC7 that derived from a patient with MM in progression and unresponsive to chemotherapy. In line with results on EA, IFN-γ induced PD-L1 and HLA-I expression on MMEC6 and MMEC7. Interestingly however, on MMEC8 derived from a patient in relapse IFN-γ increased PD-L1 but not HLA-I expression.

DISCUSSION

Multiple Myeloma remains an incurable disease and novel therapeutic strategies did not lead to a survival higher than 5 years in the adult [41]. Some benefits have been achieved using drugs such as lenalidomide that can exert several immunomodulatory effects, including the exacerbation of NK cell-mediated cytotoxicity against MM [42]. *In vitro* studies and clinical trials have also explored the possible efficacy of novel strategies combining standard treatments with emerging therapies aimed to interrupt receptors/ligands interactions such as PD1/PD-L1 [43] or KIRs/HLA-I [44], which are capable of limiting the anti-tumor function of immune effectors. NKp46, NKG2D and DNAM-1 activating receptors recognize specific ligands on MM cell surface and unchain the anti-MM activity of NK cells [45, 46]. In particular, as for other malignancies [21, 22], a pivotal role in the NK-mediated aggression of MM is played by DNAM-1/PVR interactions. These are required for optimal anti-MM efficacy of standard therapies based on the administration of cyclophosphamide and bortezomib. Moreover, it has been shown that different classes of therapeutic agents upregulate DNAM-1 as well as

NKG2D ligands [47, 48], thus strengthening the concept that NK cells might truly represent powerful adjuvant arms against MM.

Our present study suggests that NK cell-based immunotherapy might also be effective in MM patients because of the capability of NK cells to kill tumor-associated endothelial cells, which are involved in neo-angiogenesis and represent a source of soluble factors involved in paracrine loops mediating plasma cell proliferation and spread [49, 50]. We showed that activated NK cells efficiently killed MMECs (and MGECs) thanks to the cooperation of multiple triggering receptors. These include NKp30 whose known ligand B7-H6, however, is virtually absent on tumor endothelium, suggesting the possible existence of novel unidentified NKp30 specific ligand/s. NKG2D ligands were weakly expressed on MMECs surface, whereas all MMECs analyzed expressed good levels of both PVR and Nectin-2, ligands of DNAM that clearly contributed to killing of MMECs. A striking exception was represented by MMEC1 and MMEC2 that resulted poorly susceptible to lysis despite the expression of high levels of PVR. DNAM-1/PVR interaction is crucial for recognition and killing of different tumors [3]. It is of note however that its action could be counteracted by the engagement of TIGIT [20, 51] an inhibitory receptor which recognizes nectin-3 as well as PVR and nectin-2, with higher affinity than DNAM-1 [51]. Thus the different susceptibility to killing of PVR^{high} MMECs might be

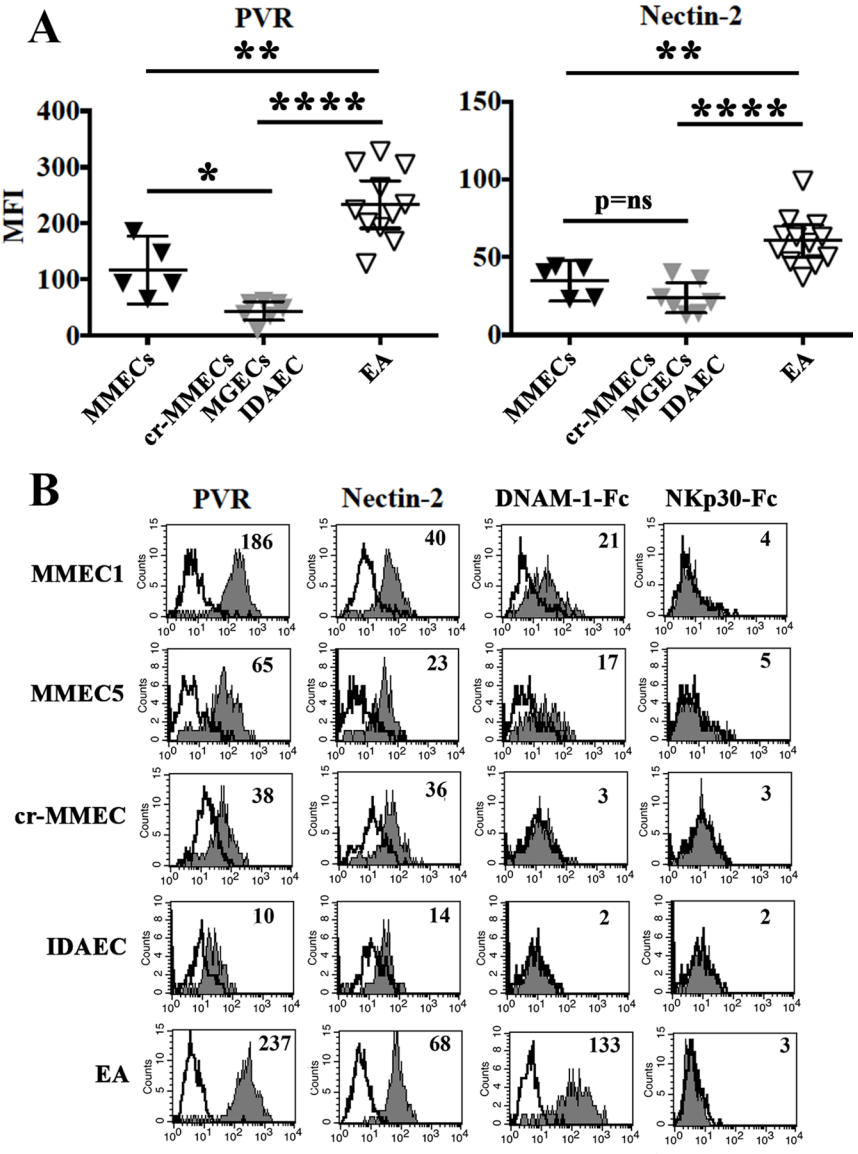

Figure 2: PVR and nectin-2 expression in MMECs and normal endothelial cells. (**A**) MMECs (black triangles), cr-MMEC, MGECs, IDAEC (gray triangles) and EA (white triangles) were analyzed by flow cytometry for the expression of PVR and Nectin-2. Mean and 95% confidence intervals are indicated. *$p < 0.05$, **$p < 0.0001$, ****$p < 0.001$, p = ns means not significant. (**B**) Representative cytofluorimetric analysis of PVR, Nectin-2, DNAM-1-Fc and NKp30-Fc staining in MMECs, cr-MMEC, IDAEC and EA. White profiles refer to cells incubated with isotype-matched mAbs. Value inside each histogram indicates the Median Fluorescence Intensity (MFI).

linked to different combinations of PVR-specific paired receptors on NK cells [51]. Another possibility is that in some patients the function of TIGIT may dominate due to the expression of nectin-3 on MMECs. Both hypotheses are currently under investigation.

In line with data obtained from the immunohistochemical analysis of primary glioblastoma specimens [8], PVR showed a high expression in tumor-associated endothelium from MM BM aspirates. In a physiological context, DNAM-1/PVR interactions have been shown to promote the transendothelial migration process of monocytes [52]. It would be of interest to understand whether the high expression of PVR in MMECs might improve migration of monocytes, which in the tumor microenvironment tend to differentiate into macrophages and acquire a M2 tumor-promoting functional polarization [53, 54]. Moreover, PVR expression might directly favor the function of MMECs thus contributing to the formation

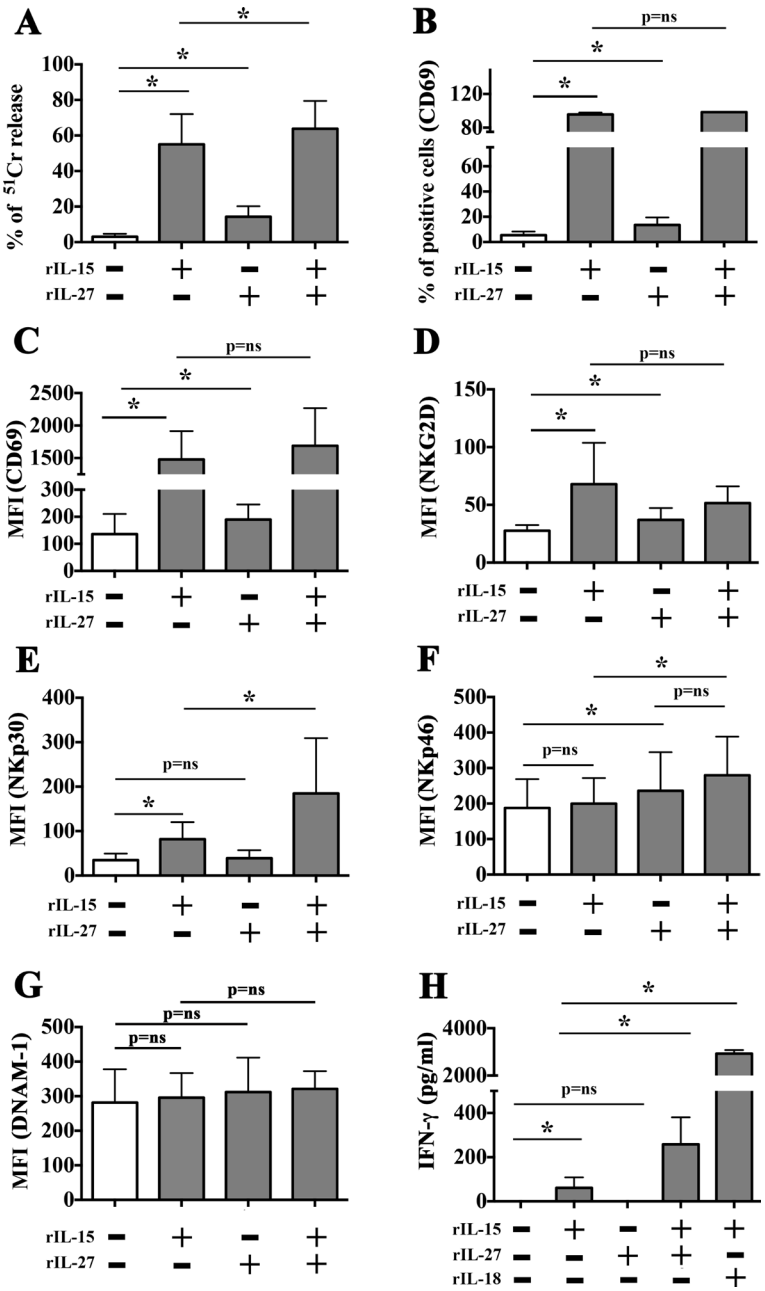

Figure 3: Comparison between rIL-27 and rIL-15-mediated stimulation of resting NK cells. NK cells purified from healthy donors were stimulated with rIL-15 (20 ng/ml) and rIL-27 (100 ng/ml) used alone or in combination. Cells were analyzed for (**A**) cytotoxicity against EA cells (^{51}Cr release assay, E:T ratio 20:1), (**B**–**G**) expression of the indicated surface molecules (flow cytometry). Culture supernatants were analyzed for the presence of IFN-γ (ELISA assay) (**H**). Mean and 95% confidence intervals are indicated. *$p < 0.05$, p = ns means not significant. Data shown are pooled from 6 independent experiments performed using NK cells from 6 unrelated healthy donors.

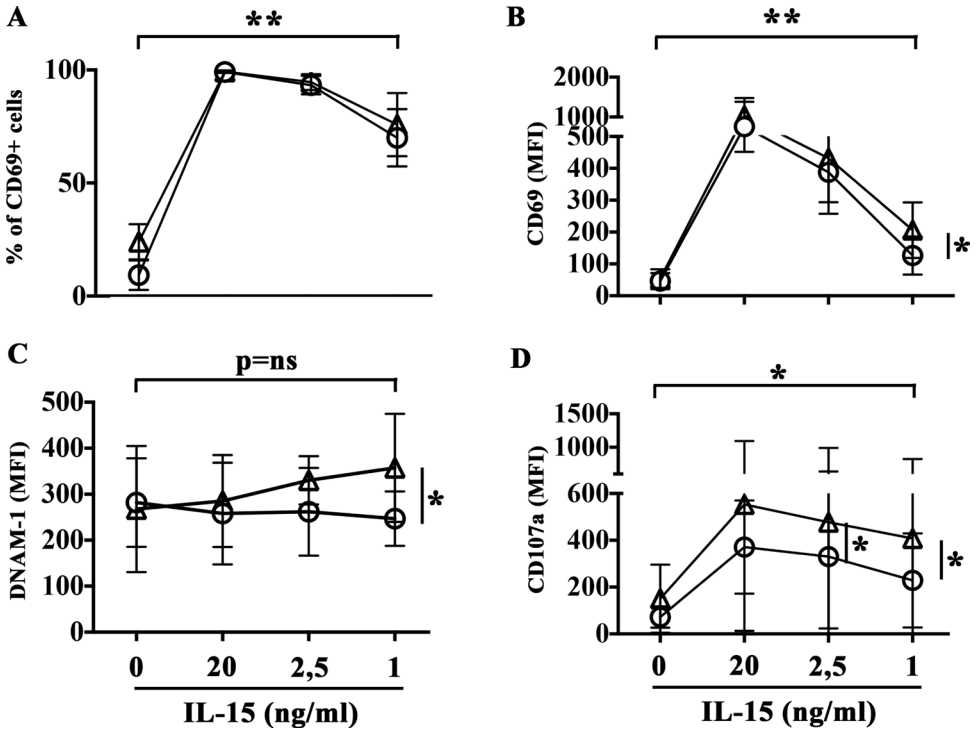

Figure 4: rIL-27 plus suboptimal doses of rIL-15 induce NK cell activation. NK cells purified from healthy donors were stimulated with decreasing concentrations of rIL-15 alone (circle) or in combination with rIL-27 (100 ng/ml) (triangle). Cells were analyzed for (**A**–**C**) surface phenotype (flow cytometry), (**D**) release of cytotoxic granules in the presence of EA cells (CD107a assay). Mean and 95% confidence intervals are indicated. $*p < 0.05$, $**p < 0.01$. p = ns means not significant. Data shown are pooled from 6 independent experiments performed using NK cells from 6 unrelated healthy donors.

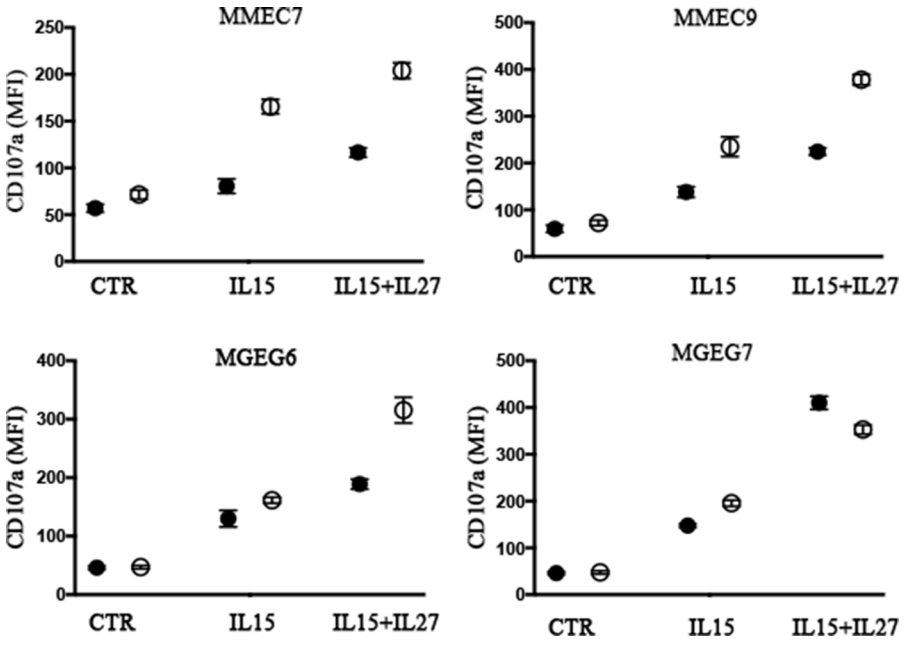

Figure 5: NK cell degranulation in the presence of MMECs and MGECs is increased by rIL-27 and suboptimal concentrations of rIL-15. NK cell populations derived from 2 healthy donors (black and white circles) were stimulated with suboptimal concentrations (1 ng/ml) of rIL-15 alone or in combination with rIL-27 (100 ng/ml). Cells were analyzed for degranulation (CD107a assay) in the presence of the indicated MMECs and MGECs. Controls (CTR) represent NK cell degranulation of NK cells stimulated with rIL-15 and rIL-27 in the absence of target. Data shown are pooled from 2 independent experiments. Mean are indicated.

of the complex architecture of tumor vasculature. In this context, different studies showed that PVR improve tumor cell invasion, being localized at the migrating cellular front together with actin and alphav-integrin, known mediators of motility and adhesion [55–57]. Thus, it should be taken into consideration that therapies upregulating PVR expression could on one side potentiate the NK cell-mediated killing by improving the formation of effective immune synapses, on the other they may favor functional capabilities of MM and MMECs.

DNAM-1/PVR interactions also occur during the crosstalk between NK cells and Dendritic cells (DC) [58, 59] or Macrophages [60, 61]. During their activation these antigen presenting cells (APC) release different cytokines capable of amplifying NK (and T) cell-mediated responses. These include IL-27 that is of interest in tumor immunology because it combines immunostimulatory and anti-angiogenic properties [30, 31]. We have analyzed the effects of IL-27 on NK cell functions, using the cytokine alone or in combination with IL-15. IL-15 represents a promising immunostimulatory adjuvant for therapies [62, 63], and a clinical grade formulation is currently available. However, different data suggested that IL-15 is also endowed with strong pro-angiogenic effects [64, 65]. According to previous data, we showed that optimal doses of IL-15 efficiently stimulate NK cells and increase their killing of MMECs. Interestingly, a similar cytolytic potential was observed in NK cells stimulated with IL-27 and suboptimal doses of IL-15 ($IL\text{-}15^{1\ ng}$ and $IL\text{-}27^{100\ ng}$). This effect was paralleled by a trend toward the increased expression of DNAM-1, NKp30, NKG2D and NKp46 activating receptors. In this context, and according with previous published data [66], the upregulation of NKp46 in NK cells was mainly IL-27-dependent.

In some instances, IL-27 did not ameliorate the effect of optimal doses of IL-15. For example, a significant increase of DNAM-1 expression could be appreciated only when IL-27 was used in combination with suboptimal doses of IL-15 (see Figures 3 and 4). This observation could be partially due to the fact that, in NK cells, IL-15- and IL-27-mediated signals share some transducing molecules such as JAK1 and STAT5 [67, 68]. Moreover, IL-15 might play a role in regulating IL-27R expression and/or function (under investigation).

NK cells stimulated with IL-27 and suboptimal doses of IL-15, release negligible amounts of IFN-γ, which is considered the best inducer of HLA-I molecules as well as of the PD-Ls immune-checkpoints ligands [34].

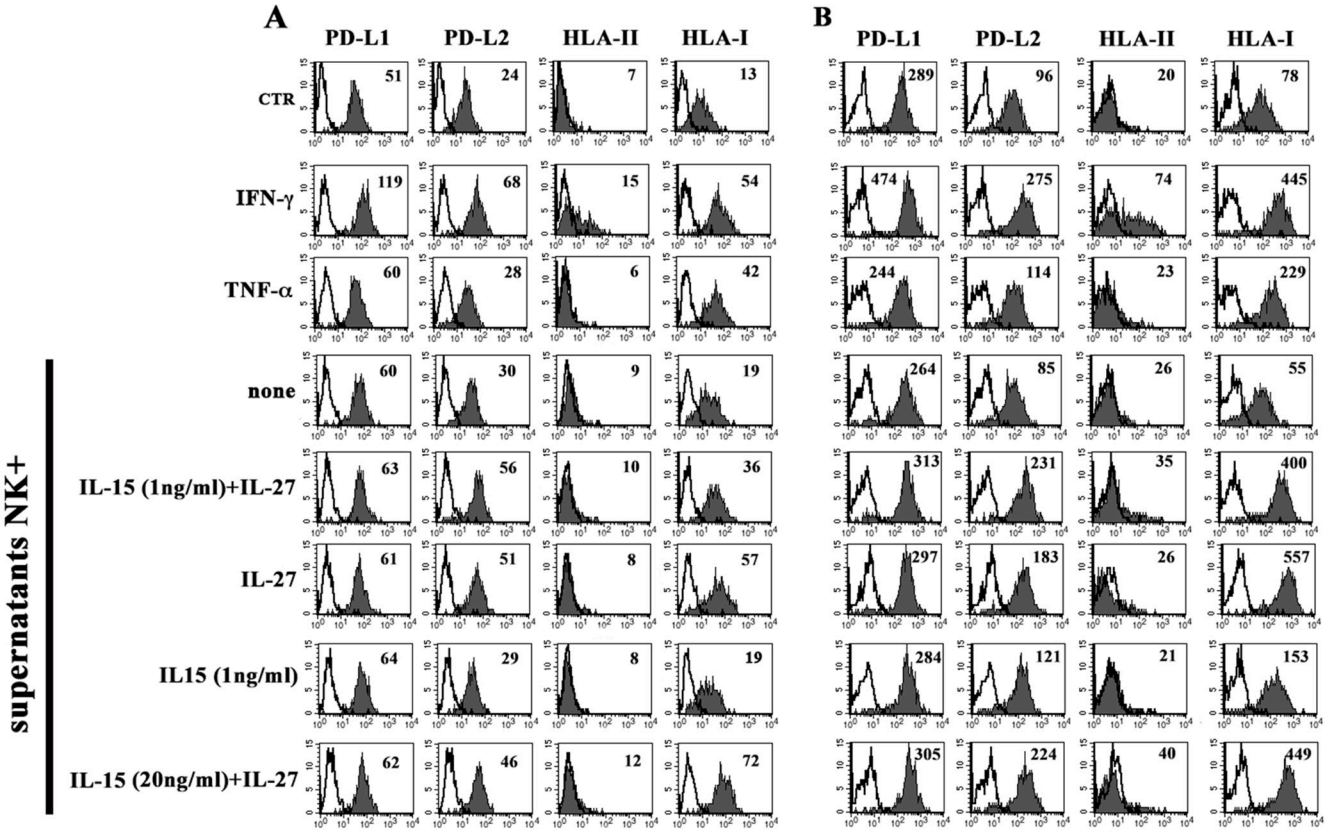

Figure 6: Constitutive or inducible expression of PD-Ls, HLA-II and HLA-I in EA cell line. The EA cell line untreated (CTR), treated with IFN-γ, TNF-α or culture supernatants from NK cells unstimulated (none) or stimulated with the indicated cytokines, was analyzed by flow cytometry for the expression of PD-Ls, HLA-II and HLA-I. Panel **A** and **B** show two independent experiments performed using NK cells purified from two unrelated healthy donors. White profiles refer to cells incubated with isotype-matched mAbs. Values inside each histogram indicate the MFI.

In EA and MMECs, IL-27 (either alone or in combination with IL-15^{1ng}) did not upregulate the expression of PD-L1 or induce that of HLA-II. On the other hand, it upregulated the expression of HLA-I, as previously demonstrated in normal endothelium (HUVEC cells) [69]. Moreover our study highlighted a peculiar function of this cytokine, i.e. the capability of upregulating the expression of PD-L2 on tumor endothelium. It is of note that previous published data suggest that IL-27 function might vary depending on target cell type and cytokine milieu. Indeed, IL-27 has been shown to drive upregulation of HLA-I also in chronic eczema keratinocytes that produced IL-27, but it promoted PD-L1 expression in different cell types including CD4+ and CD8+ T cells, monocytes, DC and tumor cells [29, 30, 70]. Thus, similar to IFN-γ (and TNF-α), IL-27 might exert possible side effects modulating the expression in MMECs of HLA-I, which downregulates the NK cell function, and PD-L2 that controls the duration and amplitude of both CD8+ T and NK cell functions. In this context, it has been shown that in MM patients more than 50% of peripheral blood NK cells express PD-1 [43], the PD-Ls receptor, although at levels lower than PD1^{+} NK cells detected in normal individuals or in different pathological conditions [72]. Thus, standard or emerging therapeutic approaches should not disregard the possible induction of a cytokine storm that might shape immune responses against tumor cells and/or tumor-associated endothelium. As for IFN-γ [71] the IL-27 capability of upregulating immune checkpoint ligands on tumor endothelium, may greatly vary among patients. Notably, during anti-tumor immune responses IL-27 may act earlier than IFN-γ, being a cytokine produced by innate cells such as macrophages and DC.

MATERIALS AND METHODS

Patients

Bone marrow (BM) aspirates were obtained from nine MM patients fulfilling the International Myeloma Working Group diagnostic criteria for multiple myeloma (MM). MM patients, enrolled at diagnosis (MM1-6, MM9), in progression because unresponsive to therapy (MM7), in relapse (MM8), were characterized by active, symptomatic disease (D&S stage II-III). Additional BM aspirates were obtained from a MM patient in complete/objective remission (cr-MM), seven patients with monoclonal gammopathy of undetermined significance (MGUS), and a patient with anemia due to iron deficiency (IDA). Approval from the Ethics Board was obtained (N°4220/2013), and patients were asked to provide their written informed consent in accordance with the Declaration of Helsinki.

Cells used in the study

Primary endothelial cells (ECs) were obtained from BM of patients as described [37]. Briefly, ECs were purified from BM-derived mononuclear cells by immunoselection, using magnetic microbeads (Dynal, Oslo, Norway) coated with Ulex europaeus agglutinin-1 lectin (UEA-1, Sigma Chemical), whose receptor is selectively and highly expressed by endothelial cells. ECs bound to microbeads were cultured in complete DMEM medium supplemented with 20% of heat-inactivated fetal bovine serum (FBS) to allow cells to adhere, spread and grow. Endothelial cells were growth

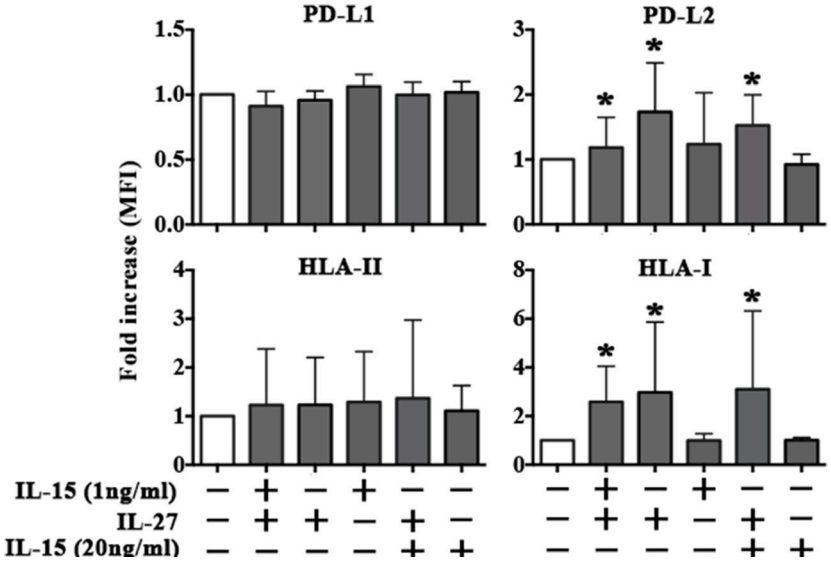

Figure 7: rIL-27 upregulates PD-L2 and HLA-I in EA cell line. The EA cell line untreated (white bar) or treated with rIL-15 and rIL-27 alone or in combination (gray bars) was analyzed by flow cytometry for the expression of PD-Ls, HLA-II and HLA-I. MFI fold increase is shown. Mean and 95% confidence intervals are indicated. *$p < 0.05$. Data shown are pooled from 4 independent experiments.

for at least one passage and their purity was confirmed by flow cytometry (FACScalibur, Becton Dickinson and Co, Mountain View, CA) analyzing the presence of the ECs markers factor VIII–related antigen (anti-human Von Willebrand factor mAb, Beckman Coulter, Marseille, France) and CD105 (anti-human CD105 mAb, Beckman Coulter), and the absence of CD14 (anti-human CD14 mAb, Becton Dickinson) and CD38 (anti-human CD38 mAb, Becton Dickinson) molecules. ECs viability was assessed by trypan blue exclusion staining (> 97% viable cells). The human endothelial (EA.hy926, henceforth named EA) and erythroleukemia (K562) cell lines were purchased from American Type Culture Collection (ATCC).

After approval by the Ethics Board (N°39/2012) and informed consent, buffy coats were obtained from healthy volunteer blood donors admitted at the transfusion center of IRCCS AOU San Martino-IST (Genova, Italy). Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Hypaque gradients and frozen at –80°C. Upon arrival of MMECs in the laboratory by express courier, PBMC were thawed and either stimulated with cytokines or used to purify NK cells (Human NK Cell Isolation kit, Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) as previously described [53]. The degree of purity of the isolated NK cells ($CD3^-$, $CD56^+$, $NKp46^+$) was superior to 98%.

Monoclonal antibodies and cytokines

The following mAbs were produced in our laboratory: A6136 (IgM) and 6A4 (IgG1) (anti-HLA class I-A, -B, -C and HLA-E), FST24 (IgG2b, anti-HLA-II), A6/220 (IgM, anti-CD56), BAB281 (IgG1) and KL247 (IgM) (anti-NKp46), AZ20 (IgG1) and F252 (IgM) (anti-NKp30), BAT221 (IgG1, anti-NKG2D), KRA236 (IgG1) and F5 (IgM) (anti-DNAM-1), BAM195 (IgG1, anti-MICA), M5A10 (IgG1, anti-PVR), U191 (IgM, anti-Nectin-2), c227 (IgG1, anti-CD69). MAB1380 (IgG2a, anti-ULBP1), MAB163903 (IgG2A, anti-ULBP2), MAB1517 (IgG2A,

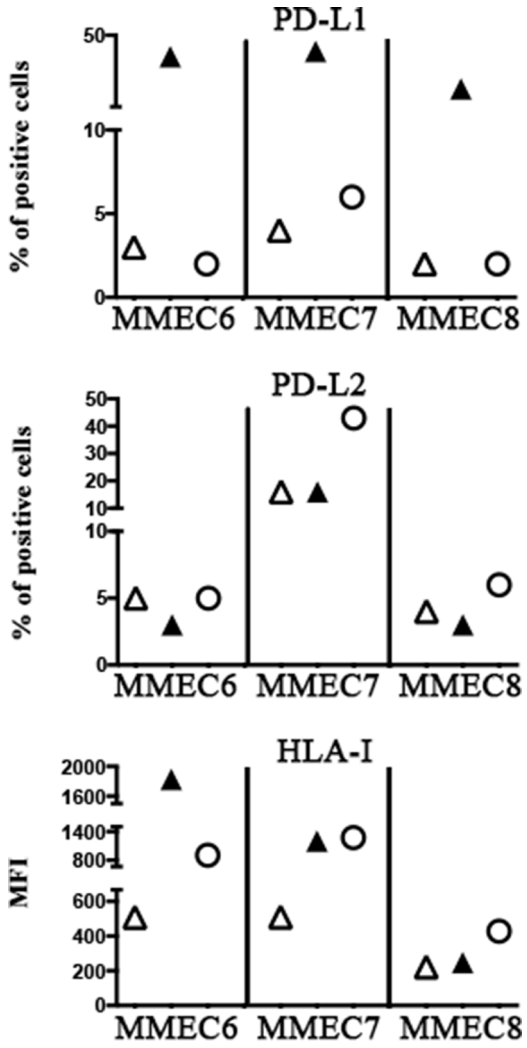

Figure 8: Effect of rIL-27 on PD-Ls and HLA-I expression in MMECs. Three MMECs either untreated (open triangle) or treated for 5 days with IFN-γ (black triangle) or IL-27 (open circle) were analyzed by flow cytometry for the expression of PD-Ls and HLA-I. Percentage of positive cells or MFI is indicated.

anti-ULBP3) and M475 (IgG2B, anti-ULBP4) mAbs were purchased from R&D System Inc., (Minneapolis, MN, USA); anti-CD107a-PE and anti-CD56-PC5 mAbs were purchased from Becton Dickinson. Anti-PD-L1.3.1 (IgG1, anti-PD-L1) and anti-PD-L2 (IgG1, anti-PD-L2) were generated in D. Olive's lab.

Human recombinant cytokines were purchased from PeproTech (rIL-15, rIFN-γ and rTNFα) MBL International (rIL-18) and R&D Systems (rIL-27).

Flow cytometry, cytolytic and ELISA assays

For one-color immunofluorescence and cytofluorimetric analysis (FACSCalibur Becton Dickinson) cells were stained with the appropriate mAbs or isotype matched controls followed by PE-isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associated, Birmingham, AL). On every experimental session, the flow cytometer performances were monitored and the reproducibility of the fluorescence intensity was aligned by calibrated microsphere (Becton Dickinson). ECs were gated on the basis of physical parameters (SSC = Side Scatter; FSC = Forward scatter) to detect viable cells. To verify the appropriateness of the gating strategy cells were stained (10 minutes at the room temperature) with Annexin V and To-Pro-3 Iodide (Life Technologies, CA, USA).

Purified NK cells were cultured for 2 days in the presence of recombinant cytokines to obtain polyclonal activated NK cell populations, which were analyzed for their cytolytic activity against target cells using a 4 h ^{51}Cr-release assay [21]. For CD107a (LAMP-1) degranulation assay NK cells were incubated for three hours with target cells in the presence of a PE-conjugated anti-human CD107a (IgG1; BD Biosciences).

The IFN-γ enzyme-linked immunosorbent human assay (ELISA) was performed according to the manufacturer's instruction (Life Technologies).

Cell transfectants and chimeric receptors

The BW5147/B7-H6+ stable cell transfectant was prepared by retrovirus gene transfer using a B7-H6 ORF cDNA obtained by RT-PCR from the MM6 human myelomonocytic cell line and subcloned in pMXs-IG (IRES-GFP) retrovirus vector (kindly provided by Dr. Kitamura, Tokyo, Japan). The empty vector pMXs-IG was used to generate the negative control (mock cell transfectant). DNAM1-Fc and NKp30-Fc were obtained in our lab fusing the extracellular region of the receptor with a mutated human IgG1 Fc portion that lack the ability to bind FcRs [52, 73].

Real-time PCR

Total RNA was extracted from K562 and EA cell lines and from immature dendritic cells (iDC) using RNAeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Starting from 1 μg RNA, oligodT-primed cDNA was prepared using Transcriptor First Strand Synthesis Kit (Roche diagnostic, Mannheim, Germany). The expression of B7-H6 and HPRT1 (hypoxanthine phosphoribosyltransferase 1) transcripts was assessed by real-time PCR with the Taqman Gene expression assays (Hs02340611_m1 and Hs99999909_m1, respectively) and Express qPCR SuperMix (Thermo Fisher Scientific, Waltham, MA, USA). Samples were run on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). Each reaction was performed in triplicate and each sample was analysed in three independent experiments. Relative expression of B7-H6 transcript was determined in each sample by normalization with respect to the HPRT1 gene, according to the standard ΔC_T method.

Statistical analysis

Statistical analysis with level of significance (p) and graphic representation were performed using Wilcoxon-Mann-Whiteny *p*-value test (non-parametric significance test) and GraphPad Prism 6 (GraphPad Software La Jolla, CA).

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CONFLICTS OF INTEREST

D.Olive is co-founder of Imcheck Therapeutics (Marseille, France). A. Moretta is founder and shareholder of Innate Pharma (Marseille, France). The remaining authors declare no conflicts of interest.

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TGF-β1 Downregulates the Expression of CX_3CR1 by Inducing miR-27a-5p in Primary Human NK Cells

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Activity of human natural killer (NK) cells against cancer cells is deeply suppressed by TGF-β1, an immunomodulatory cytokine that is released and activated in the tumor microenvironment. Moreover, our previous data showed that TGF-β1 modifies the chemokine receptor repertoire of NK cells. In particular, it decreases the expression of CX_3CR1 that drives these effectors toward peripheral tissues, including tumor sites. To identify possible mechanisms mediating chemokine receptors modulation, we analyzed the microRNA profile of TGF-β1-treated primary NK cells. The analysis pointed out miR-27a-5p as a possible modulator of CX_3CR1. We demonstrated the functional interaction of miR-27a-5p with the 3′ untranslated region (3′UTR) of CX_3CR1 mRNA by two different experimental approaches: by the use of a luciferase assay based on a reporter construct containing the CX_3CR1 3′UTR and by transfection of primary NK cells with a miR-27a-5p inhibitor. We also showed that the TGF-β1-mediated increase of miR-27a-5p expression is a consequence of miR-23a-27a-24-2 cluster induction. Moreover, we demonstrated that miR-27a-5p downregulates the surface expression of CX_3CR1. Finally, we showed that neuroblastoma cells induced in resting NK cells a downregulation of the CX_3CR1 expression that was paralleled by a significant increase of miR-27a-5p expression. Therefore, the present study highlights miR-27a-5p as a pivotal TGF-β1-induced regulator of CX_3CR1 expression.

Keywords: TGF-β1, chemokine receptors, microRNAs, NK cells, tumor microenvironment

INTRODUCTION

Human natural killer (NK) cells mainly consist of $CD56^{bright}$ $CD16^{low/neg}$ KIR^{neg} $perforin^{low}$ and $CD56^{dim}$ $CD16^{pos}$ KIR^{pos} $perforin^{high}$ cells, which represent sequential stages of maturation and NK subsets with different function and tissue distribution (1, 2). $CD56^{bright}$ NKs, which release large amount of soluble factors (IFN-γ, GM-CSF, and TNF-α) in response to proinflammatory cytokines, are poorly represented in peripheral blood, while populating most tissues, and, in particular, secondary lymphoid organs (SLOs) (3, 4). Conversely, $CD56^{dim}$ cells, which are highly cytotoxic effectors,

represent the largest percentage of circulating NKs, while being detectable only in selected peripheral tissues such as lung and breast (1). Notably, upon appropriate stimulation, $CD56^{dim}$ cells increase the cytolytic potential, become cytokines producers, and also acquire the capability to migrate to SLO (5, 6).

The NK cells distribution in physiological and pathological conditions is dictated by the expression of chemokine receptors that retain or drive the migration toward tissues of one or another NK cell subset. Peripheral blood (resting) $CD56^{dim}$ and $CD56^{bright}$ NK cells share the expression of CXCR4 (CXCL12 receptor) (7) and CXCR3 (CXCL4, 9, 10, 11 receptor). $CD56^{bright}$ NKs selectively express CCR5 (CCL3-5 receptor) and CCR7 that guides their migration toward tissues with high levels of CCL19 and CCL21, such as SLO. Conversely, the expression of CXCR1 (CXCL8 receptor), CXCR2 (CXCL1, 2, 3, 5, 8 receptor), and CX_3CR1 (CX_3CL1 receptor) is restricted to $CD56^{dim}$ NKs (4), which are also characterized by the peculiar expression of the ChemR23 specific for chemerin (8).

CX_3CL1, also termed fractalkine, is the only known member of the CX_3C chemokine family. It is synthesized as a membrane anchored molecule expressed by cells such as endothelial cells, epithelial cells, osteoblasts, and mesenchymal stromal cells. CX_3CL1 can be shed by A Disintegrin And Metalloproteinases (ADAM) 10 or 17 in non-inflammatory and inflammatory conditions, respectively (9). Once released, it interacts with the specific receptor (CX_3CR1), a molecule belonging to the 7-transmembrane G protein-coupled receptor family, which is expressed by different immune cell types, including monocytes, dendritic cells (DCs), T, and NK lymphocytes.

Tumor tissues set up different strategies to escape immune recognition and in particular to affect chemokine/chemokine receptor axes such as CX_3CL1/CX_3CR1, which are crucial for the recruitment of cells such as $CD56^{dim}$ NK cells exerting tumor suppressive properties. A possible strategy is to unbalance in the tumor microenvironment the equilibrium between $CD56^{dim}$ and $CD56^{bright}$ attracting chemokines. Immature, poor cytolytic $CD56^{bright}$ cells represent the major NK subset present in most tumor tissues (4, 10), and breast carcinomas have been shown to decrease the expression of CXCL2 and CX_3CL1 while increasing that of CCL5 and CCL19 (1). Accordingly, CX_3CL1 expression in breast carcinoma specimens was shown to correlate with a good prognosis (11).

Another possible mechanism exerted by tumors for limiting immune surveillance is represented by their capability of modifying the chemokine receptor repertoire of crucial effector cells, thus affecting their responsiveness to given chemoattractant gradients. Among the soluble mediators involved, TGF-β1 shows a pivotal role in chemokine receptors modulation (12). TGF-β1 is pleiotropic cytokine whose major physiologic role is to limit the duration of immune responses and promote tissue repair (13, 14). In the tumor microenvironment, its release and activation by cancer cells and immune cells, including tumor-associated macrophages (TAM), is exacerbated (9, 15). This results in tumor-promoting side effects including angiogenesis and suppression of anticancer immunity. In NK cells, tumor-derived and recombinant TGF-β1 has been shown to downregulate the expression of NKp30 and NKG2D activating receptors involved in tumor recognition and DC editing (16, 17) and to modulate the chemokine receptor repertoire. In particular, TGF-β1 increases the expression of CXCR3 and CXCR4 (12, 18), involved in peripheral tissues and bone marrow (BM) recruitment, respectively, and decreases that of CX_3CR1, which drives effector cells toward peripheral tissues, including central nervous system (CNS) (19–21). Interestingly, in glioma cells TGF-β1 has also been shown to downregulate CX_3CL1 (22). The presence of $CD56^{dim}$ NK cells, characterized by low expression of CX_3CR1, has been detected in ascitic fluids of ovarian carcinoma patients (10). Moreover, a possible systemic effect has been demonstrated in patients with high-risk (stage 4 or M) neuroblastoma (NB), who were characterized by the presence of unconventional $CD56^{dim}$ CX_3CR1^{low} NKs, both in metastatic sites, such as BM and peripheral blood (12).

Understanding how TGF-β1 impairs the expression of CX_3CR1 in $CD56^{dim}$ NK has potential clinical implications, since it could improve the current knowledge of the mechanisms that regulate the immune responses and, in cancer, participate in the escape from immune surveillance. MicroRNAs (miRNAs), small non-coding RNA molecules, are key players in the regulation of gene expression and modulate several biological processes, including immune responses. In this study, we performed a profile of NKs to identify miRNA that could play roles in TGF-β1-mediated modulation of chemokine receptors expression. We identified miR-27a-5p as a negative regulator of CX_3CR1 expression.

MATERIALS AND METHODS

NK Cells Purification

NK cells were purified from peripheral blood mononuclear cells of healthy donors using the Human NK Cell Isolation kit (Miltenyi Biotec) (12). A total of 13 unrelated healthy donors were used for all the experiments described. Donors provided an informed consent according to the procedures approved by the Ethics Committee of Ospedale Policlinico San Martino (39/2012).

NK Cell Treatments for miRNA and mRNA Expression Analysis

To obtain miRNA profiling, NK cells were incubated for 24 h in the presence of complete medium (RPMI 1640 with 10% of fetal bovine serum, 2 mM glutamine, 50 mg/mL penicillin and 50 mg/mL streptomycin) supplemented with TGF-β1 at the final concentration of 40 or 5 ng/mL. Control cells were represented by NK cells cultured in the presence of complete medium alone (12). For miRNA validation and mRNA expression analysis, NK cells were incubated in the presence of 40 or 5 ng/mL of TGF-β1 or complete medium for 12 or 24 h.

RNA Isolation

RNA containing the small RNA fraction was extracted using the miRCURY RNA Isolation Kit—Cell and Plant (Exiqon) according to the manufacturer guidelines. RNA amount was determined using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen) following to the manufacturer instructions.

miRNA Profiling

miRNA profiling was performed according to the TaqMan MicroRNA Array Workflow (Applied Biosystems). Briefly, 100 ng of RNA comprehensive of the small RNA fraction were reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit with the Megaplex RT Primers Human Pool A + B set v3.0 (Applied Biosystems). A preamplification step was performed using the TaqMan PreAmp Master Mix with the Megaplex PreAmp Primers Human Pool A + B set v.3.0 (Applied Biosystems). Preamplified cDNAs were loaded in TaqMan Array Human MicroRNA Cards A and B set v3.0 (Applied Biosystems). Amplification reactions were performed using an Applied Biosystems ViiA 7 Real-Time PCR System. Data were analyzed using the miScript miRNA PCR Array Data Analysis Tool.[1] miRNAs expression was normalized to the U6 snRNA expression. Data from this miRNA profiling have been submitted to the NCBI Gene Expression Omnibus[2] under accession no. GSE98769. miRNAs that showed a fold change >2 or <0.5 between TGF-β1-treated and -untreated NK cells in three different donors were considered to be differentially expressed. Using the computational prediction on-line tools TargetScan[3] (23), miRanda[4] (24), and miRmap[5] (25), selected miRNAs were investigated as possible regulators of CXCR4, CXCR3, and CX_3CR1 expression.

Expression of miRNAs

Expression of selected miRNAs was evaluated using TaqMan MicroRNA Assays as described by the manufacturer (Applied Biosystems). Briefly, 10 ng of RNA were reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit primed with the specific RT primers. Real-time PCR was performed in quadruplicate using the specific primers. Expression of each miRNA was normalized to the RNU44 expression.

Expression of CX_3CR1 mRNA

To evaluate the expression of CX_3CR1 mRNAs, 100 ng of RNA were reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). The cDNA was used for real-time PCR using the specific primers contained in the TaqMan Gene Expression Assay (Applied Biosystems). CX_3CR1 expression was normalized to the GAPDH expression. Experiments were performed in quadruplicate.

CX_3CR1 3′ Untranslated Region Construct

A 700 bp fragment of the CX_3CR1 3′ untraslated region (3′UTR) containing the putative target site for miR-27a-5p was amplified by PCR using primers CX3UTR2F and CX3UTR2R, containing the NheI and XhoI sites in their 5′ ends, respectively. The PCR product was purified using the MinElute PCR Purification Kit (Qiagen), NheI-XhoI digested and ligated to the NheI-XhoI digested pmirGLO vector (Promega) using the Rapid DNA Ligation kit (Roche, Basel, Switzerland). Ligation product was used for transformation of TOP10 *Escherichia coli* competent cells (Invitrogen). Colonies containing the recombinant plasmid were selected by PCR using primers CX3UTR2F/CX3UTR2R. Plasmid DNA was purified using the Illustra PlasmidPrep Mini Spin Kit (GE Healthcare). The inserted fragment and flanking sequences were sequenced using primers CX3UTR2F, CX3UTR2R, CX3UTR22R2, and CX3UTR22F2. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in a 3100 Genetic Analyzer (Applied Biosystems). The selected plasmid is reported as pmirCX-3UTRWT. Primers, when not otherwise indicated, were designed by primerBLAST.[6] Primer sequences are reported in Figure S1 in Supplementary Material.

Site-Directed Mutagenesis

A mutated version of pmirCX3UTRWT (pmirCX3UTRMT), containing a C>G mutation in the putative target site for miR-27a-5p, was prepared by site-directed mutagenesis using the Geneart Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer guidelines. Oligonucleotides (CX3MUTF and CX3MUTR) were designed using the QuickChange Primer Design on-line tool (Agilent Technologies[7]). Presence of the mutation was confirmed by restriction analysis and by sequencing the mutagenized region using primers CX3UTR2FS and CX3UTR22R2.

Luciferase Reporter Assay

Wild-type (pmirCX3UTRWT) and mutant (pmirCX3UTRMT) plasmids containing the 3′UTR of the CX_3CR1 mRNA, as well as the parental pmirGLO vector (Promega), were used in cotransfection experiments with the mirVana miRNA Mimic miR-27a-5p and the mirVana miRNA Mimic Negative Control #1 (Ambion). HEK293T cells were used for cotransfection experiments. The day before transfection 8×10^4 cells per well were plated in 24-well plates in 500 µL of DMEM supplemented with 10% FCS. Cells were transfected with 100 ng of plasmid and 20 pmol of mimic miRNA using lipofectamine 2000 (Invitrogen) according to the manufacturer protocol. Twenty-four hours post transfection cells were harvested. Firefly and Renilla Luciferase activities were determined on cell lysates using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer guidelines. A single-tube DLReady validated luminometer TD-20/20 Turner Biosystems was used. Firefly luciferase activity was normalized to renilla luciferase activity. Normalized values were expressed as changes relative to the value of the negative control, which was set as 1. Three independent experiments were performed in quadruplicate.

[1] http://www.sabiosciences.com/mirnaArrayDataAnalysis.php.
[2] http://www.ncbi.nlm.nih.gov/geo/.
[3] www.targetscan.org.
[4] www.microrna.org.
[5] http://mirmap.ezlab.org.
[6] http://www.ncbi.nlm.nih.gov/tools/primer-blast/.
[7] http://www.genomics.agilent.com.

miR-23a-27a-24-2 Cluster Primary Transcript Expression

The 1×10^6 NK cells from healthy donors were cultured in the presence or in the absence of 5 ng/mL of TGF-β1. Real-time PCR was performed using the hsa-mir-23a TaqMan Pri-miRNA Assay (Applied Biosystems). Hsa-mir-23a pri-miRNA expression was normalized to the GAPDH expression. Experiments were performed in triplicate.

Transient Transfection of NK Cells

The 1×10^6 NK cells from healthy donors were cultured in 24-well plates in 500 µL RPMI + 10% serum in the presence of TGF-β1 (5 ng/mL). Cells were transfected with 20 pmol of miR-27a-5p inhibitor (Applied Biosystems) or with a negative control (mirVana™ miRNA Inhibitor, Negative Control #1; Ambion) using Lipofectamine 3000 according to manufacturer instructions. Efficiency of transfection, determined with a fluorescently labeled miRNA (Cy3™ Dye-Labeled Pre-miR Negative Control #1; Ambion) was about 30% (data not shown). Cells were harvested after 72 h, RNA was extracted, and CX_3CR1 mRNA levels were determined as described previously.

CX3CR1-Lentiviral Vector Generation

Primer CX3ATGKZF, designed to contain a Kozak consensus sequence, and primer CX3UTR2R (Figure S1 in Supplementary Material) were used for the amplification of a genomic fragment (2,494 bp in length) containing the whole CX3CR1 gene open reading frame (ORF) and a portion of the 3′UTR in which the two putative target sites for miR-27a-5p are included. The product was cloned in a pcDNA 3.1/V5-His-TOPO vector (Invitrogen), excised by XbaI-BamHI digestion, and ligated to an XbaI-BamHI digested pCDH-CMV-MCS-EF1-GFP lentiviral vector (System Biosciences).

Ligation product was transformed in Stbl3 *E. coli* cells (Invitrogen), a positive colony was selected by PCR, endotoxin-free plasmid DNA was extracted using the QIAfilter Plasmid Midi Kit with the EndoFree Plasmid Buffer Set (Qiagen), the whole insert and the flanking vector regions were sequenced using 16 primers (whose sequences are reported in Figure S1 in Supplementary Material).

Virus Packaging

The lentiviral vector containing the CX_3CR1 gene was assembled in lentiviral particles using the pPACKH1 HIV Lentivector Packaging Kit (System Biosciences). Briefly, 3×10^6 293T cells plated in a 10 cm plate were tranfected with the packaging plasmids and the CX_3CR1 lentiviral vector with lipofectamine 2000 (Invitrogen). Medium was changed every 24 h and collected at 48 and 72 h. Virus containing supernatants were pooled and concentrated by the use of a PEG precipitation solution. Titration was performed by flow cytometric detection of GFP fluorescence emitted by infected cells.

HEK293T Cells Transduction

HEK293T cells (2×10^5) were plated in six-well plates and infected with viral preparation at a multiplicity of infection of 2 in 500 µL complete medium without antibiotics in the presence of 8 µg/mL of polybrene (Santa Cruz Biotechnology, Dallas, TX, USA). Infected cells were centrifuged for 1 h at room temperature at 2,000 rpm, then incubated at 37°C. 24 h after infection, medium was removed and replaced with 4 mL of fresh medium without polybrene. One week after infection, CX_3CR1 positive cells were selected using the anti-CX_3CR1 Microbead Kit (Miltenyi Biotec). Clones generated by limited dilution of the selected cells were analyzed for CX_3CR1 surface expression by flow cytometry. Clones showing a good and stable expression of CX_3CR1 were expanded and used for transfection with miR-27a-5p and with miR-Neg.

Transfection of CX_3CR1-Expressing HEK293T Cells

An HEK293T clone stably expressing CX_3CR1 was transfected with miR-27a-5p and with miR-Neg to evaluate the modulation of CX_3CR1 expression. The 10^5 cells were plated in six-well plates in 2 mL complete medium, transfection was performed using 100 pmol of miRNA mimics and 3 µL of Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer guidelines. Cells were harvested 48 and 72 h after transfection. CX_3CR1 expression was analyzed by flow cytometry.

Coculture of NK Cells and SH-SY5Y NB Cell Line in Transwell Condition

For coculture experiments in transwell condition, 2×10^5 resting NK cells were cultured for 48 h with 3×10^5 SH-SY5Y cells. NK cells and SH-SY5Y cells were placed in 24-well transwell (0.3-µm pore size; Corning Costar), upper and bottom chamber, respectively (12).

Statistical Analysis

The statistical level of significance (p) is indicated using Student's t-test, a parametric significance test. Graphic representation and statistical analysis were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Pearson's correlation coefficient (two-tailed test) was used to evaluate the correlation between CX_3CR1 mRNA and miR-27a-5p expression.

RESULTS

miRNA Profiling of TGF-β1-Treated NK Cells

To identify miRNAs possibly involved in the TGF-β1-mediated modulation of chemokine receptors expression, we investigated the miRNA expression profile variation in TGF-β1-treated NK cells purified from the peripheral blood of three unrelated healthy donors. The analysis, allowing the simultaneous profiling of 754 miRNAs (**Figure 1**), showed that miRNAs up- or down-regulated by the treatment ranged from 127 in donor 2 to 234 in donor 3 (Figure S2 in Supplementary Material). A total of 14 miRNAs were modulated in NK cells from all donors analyzed upon treatment with either 5 or 40 ng/mL of TGF-β1. Among these 14 miRNAs, 11 were excluded from further analysis being expressed at low levels (threshold cycle <33), while 1 (miR-1201)

was forsaken due to its sequence overlapping to an annotated small nucleolar RNAs (snoRNA, SNORD126), as reported in miRBase.[8] The remaining two miRNAs (miR-302a and miR-27a-5p) were further investigated as putative regulators of CXCR4, CXCR3, or CX_3CR1 expression (**Figure 1** and Figure S2 in Supplementary Material).

[8]www.mirbase.org.

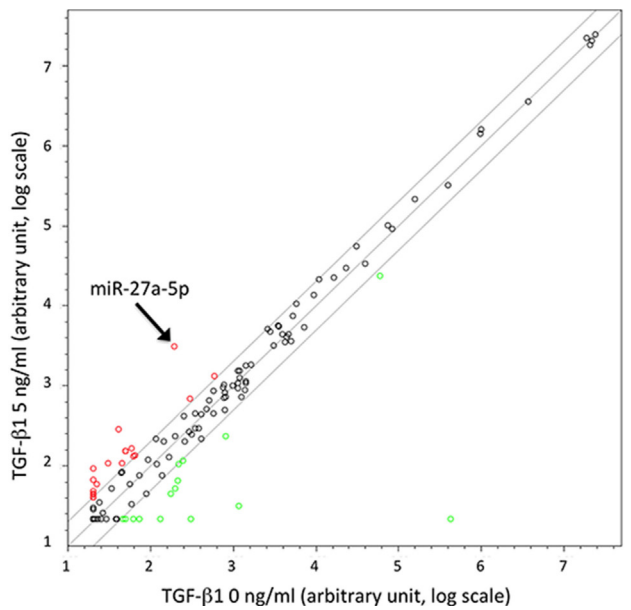

FIGURE 1 | Scatter plot showing microRNAs (miRNAs) up- or downregulated in TGF-β1-treated natural killer (NK) cells. Results are referred to NK cells from a representative healthy donor of three analyzed (donors 1, 2, and 3) treated with TGF-β1 (5 ng/mL). Plot is referred to TaqMan Array Human MicroRNA Cards B (Applied Biosystems). Results are expressed as arbitrary units on a log scale. U6 snRNA has been used as reference control. miR-27a-5p is indicated by the arrow. Diagonal lines represent the 2, 1, and 0.5 values for fold induction.

miR-27a-5p As Putative Regulator of CX_3CR1 Expression

The differential expression of miR-302a and miR-27a-5p in untreated and TGF-β1-treated NK cells was checked for validation by using specific miRNA assays. The analysis revealed that miR-302a was virtually absent in both untreated and TGF-β1-treated NK cells (data not shown). On the contrary, in agreement with data obtained by miRNA profiling, TGF-β1-treated NK cells showed a significant increase of miR-27a-5p expression, which was higher at low TGF-β1 concentration (**Figure 2**). Thus, miR-27a-5p was further investigated as putative regulator of CXCR4, CXCR3, or CX_3CR1 expression using the computational prediction on-line tools TargetScan (see text footnote 3) (23), miRanda (see text footnote 4) (24), and miRmap (see text footnote 5) (25). The analysis indicated miR-27a-5p as a putative regulator of CX_3CR1 expression with relatively high scores. The three softwares predicted the same principal site of interaction in the CX_3CR1 3′UTR with the miR-27a-5p seed region (Figure S3A in Supplementary Material). An additional site in the CX_3CR1 3′UTR, with a weaker interaction with miR-27a-5p, was predicted by miRanda and miRmap tools only (Figure S3B in Supplementary Material).

Inverse Correlation of miR-27a-5p and CX_3CR1 mRNA Expression in TGF-β1-Treated NK Cells

Since miRNAs properties include the capability of inducing degradation of targeted mRNAs, expression profiles of miR-27a-5p and CX_3CR1 mRNAs were simultaneously analyzed over time. As shown in **Figure 3A**, at both concentrations used, TGF-β1 caused a significant increment of miR-27a-5p and decrease of CX_3CR1 mRNA expression. Importantly, expression of miR-27a-5p and CX_3CR1 mRNA was inversely correlated (Pearson's correlation coefficient $r = 0.766$, $p < 0.05$; **Figure 3B**). While preliminary data showed that 1 ng/mL of TGF-β1 may be sufficient for induction of miR-27a-5p, concentrations of 5 and 40 ng/mL were chosen for a more systematic investigation. Next, to deepen the molecular

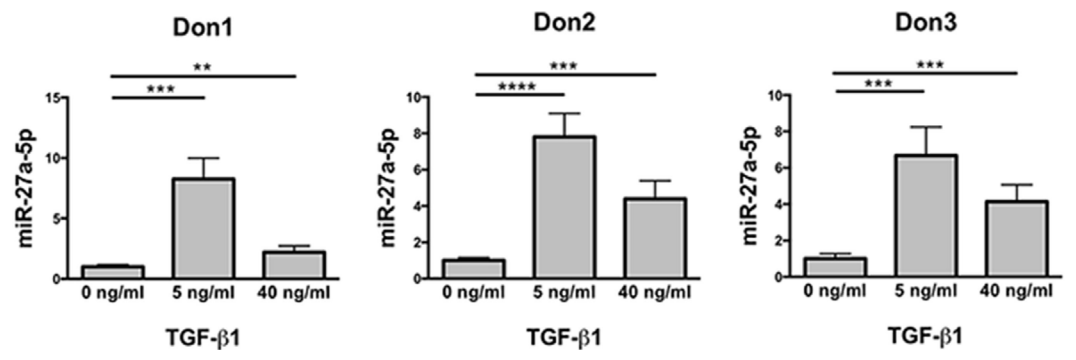

FIGURE 2 | Expression profile of miR-27a-5p in TGF-β1-treated natural killer (NK) cells. NK cells untreated or treated for 24 h with the indicated concentration of TGF-β1 were analyzed for miR-27a-5p expression. RNU44 was used as reference control. Experiments were performed in quadruplicate on NK cells derived from three healthy donors (donors 1, 2, and 3). Expression relative fold changes are referred to the expression of untreated cells, whose miR-27a-5p expression has been arbitrarily assigned the value 1. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

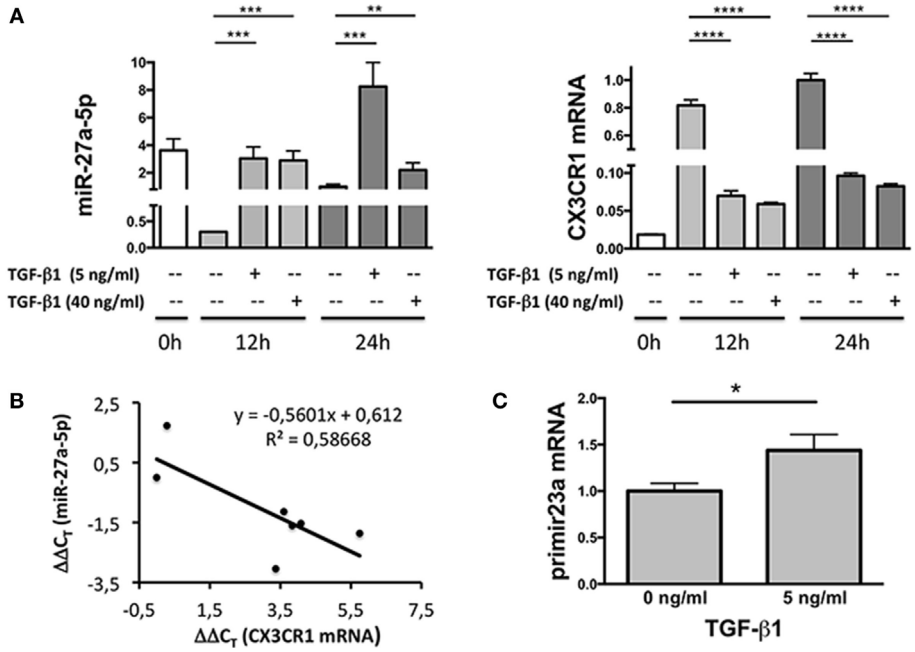

FIGURE 3 | Expression profile of miR-27a-5p and CX_3CR1 mRNA in TGF-β1-treated natural killer (NK) cells. **(A)** NK cells treated for 12 or 24 h with the indicated amounts of TGF-β1 were simultaneously analyzed for the expression of miR-27a-5p and CX_3CR1. RNU44 and GAPDH were used as reference controls, respectively. Data in quadruplicate from one representative donor (donor 1), of two analyzed (donors 1 and 4), are shown. Expression relative fold changes are referred to the expression of miR-27a-5p and CX_3CR1 mRNA in untreated NK cells (medium alone, 24 h) whose expression has been arbitrarily assigned the value 1. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. **(B)** Correlation between miR-27a-5p and CX_3CR1 mRNA expression in TGF-β1-treated NK cells. Scatter plot showing miR-27a-5p and CX_3CR1 mRNA expression in TGF-β1-treated NK cells from the same donor (donor 1) of (**A**). Each point represents the miR-27a-5p and CX_3CR1 expression detected in one of the seven reported conditions, with different TGF-β1 concentrations (0, 5, and 40 ng/mL) and time of treatment (0, 12, and 24 h). The trendline, its equation and the R^2 value is reported. Significance of correlation was determined using the Pearson's coefficient ($r = -0.765948064$, $p < 0.05$). Data are referred to a representative donor (donor 1), of two analyzed (donors 1 and 4). **(C)** miR-23a-27a-24-2 cluster primary transcript expression in TGF-β1-treated NK cells. NK cells cultured for 24 h in the presence or absence of 5 ng/mL of TGF-β1 were analyzed by real-time PCR for the expression of miR-23a~27a~24-2 cluster primary transcript. GAPDH was used as reference control. Data in triplicate from one representative donor of three analyzed (donors 5, 6, and 7) are shown. Expression relative fold changes are referred to the expression of untreated NK cells (medium alone) whose expression has been arbitrarily assigned the value 1. $^{*}p < 0.05$.

mechanism responsible for the TGF-β1-induced increase of miR-27a-5p, we analyzed the expression of the miR-23a-27a-24-2 cluster, precursor of miRNAs, including miR-27a-5p. As shown in **Figure 3C**, TGF-β1 caused a significant increment of the primary miR-23a-27a-24-2 transcript, thus demonstrating that the increased amount of miR-27a-5p might be due, at least in part, to induction of its gene expression other than, for example, to miR-27a-5p egress from intracellular stores.

miR-27a-5p Interacts with the CX_3CR1 mRNA

To unequivocally demonstrate that miR-27a-5p could interact with the 3′UTR of the CX_3CR1 mRNA, modulating its expression, we performed a luciferase reporter assay (**Figure 4**). We cloned a 700 bp fragment of the CX_3CR1 gene 3′UTR, containing all the putative sites of interaction with the seed region of miR-27a-5p, into a luciferase reporter vector, downstream of the firefly luciferase gene. Moreover, we also produced a mutated version of the construct, containing a C>G substitution in the main site of the CX_3CR1 3′UTR (Figure S3A in Supplementary Material). The two plasmids were separately cotransfected in HEK293T cells with a miR-27a-5p mimic or with a random sequence miRNA negative control. As shown in **Figure 4A**, a significant reduction of the normalized luciferase activity was detected in the presence of miR-27a-5p mimic compared to the miRNA negative control. Importantly, this effect was lost when the miR-27a-5p mimic was cotransfected with the construct having the mutated sequence of the CX_3CR1 3′UTR, thus confirming the interaction of miR-27a-5p with the CX_3CR1 3′UTR.

Next, to further confirm miR-27a-5p and CX_3CR1 mRNA interaction, before TGF-β1 treatment NK cells were transiently transfected with a specific miR-27a-5p inhibitor or with a scrambled miRNA as negative control (**Figure 4B**). As expected, we observed a significantly higher CX_3CR1 mRNA expression in TGF-β1-treated NK cells previously transfected with the miR-27a-5p inhibitor when compared with cells transfected with a scrambled miRNA.

miR-27a-5p Downregulates the Surface Expression of CX_3CR1

To determine whether miR-27a-5p is able to downregulate the surface expression of CX_3CR1, we prepared a lentiviral

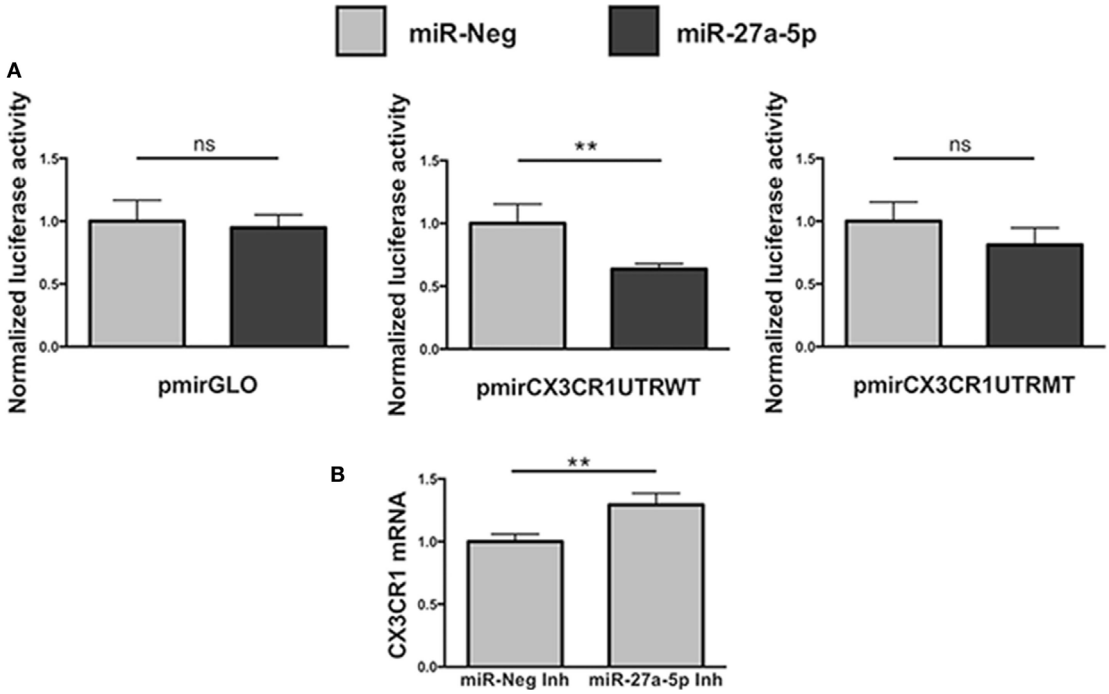

FIGURE 4 | Functional interaction between miR-27a-5p and CX_3CR1 mRNA. **(A)** Luciferase activity in HEK293T cells cotransfected with luciferase reporter construct containing the 3′ untranslated region (3′UTR) CX_3CR1 and miR-27a-5p mimics. Firefly luciferase activity was normalized to the renilla luciferase activity, expressed by the same vector. Data in quadruplicate are from one experiment of three performed. Luciferase activities are referred to the activity of cells transfected with the miRNA negative control whose expression has been arbitrarily assigned the value 1. pmirGLO, parental vector; pmirCX3CR1UTRWT, pmirGLO vector containing 700 bp of the CX_3CR1 3′UTR downstream of the firefly luciferase gene; pmirCX3CR1UTRMT, mutated version of pmirCX3CR1UTRWT containing the C>G mutation in the CX_3CR1 3′UTR target site; miR-Neg, miRNA mimic negative control. **p < 0.01; ns, not significant. **(B)** CX_3CR1 mRNA expression in NK cells transfected with miR-27a-5p inhibitor (Inh) or with a negative control miRNA Inh. Cells were cultured for 72 h in the presence of 5 ng/mL of TGF-β1. Data in triplicate are from one representative healthy donor of three analyzed (donors 8, 9, and 10). GAPDH has been used as reference control. Expression relative fold changes are referred to CX_3CR1 mRNA expression in NK cells transfected with the negative control miRNA inhibitor, whom expression has been arbitrarily assigned the value 1. *$p < 0.05$.

vector containing the whole ORF of the CX_3CR1 gene and the portion of the 3′UTR region containing the two putative binding sites for miR-27a-5p. HEK293T cells where transduced with the vector and cultured under limiting dilution to obtain CX_3CR1 positive clones. A clone (#124) was selected that stably expressed at the cell surface optimal levels of the chemokine receptor. Clone #124 was transfected with a miR-27a-5p mimic or with a miRNA negative control and analyzed by flow cytometry for the chemokine receptor expression. As shown in **Figure 5**, miR-27a-5p mimic induced a significant downregulation of the percentage of CX_3CR1 positive cells. On the contrary, the expression of other molecules such as PVR (CD155) was unaffected (Figure S4 in Supplementary Material). These data further support the role of miR-27a-5p as modulator of the chemokine receptor.

miR-27a-5p Induction in the Context of NB-NK Cocultures

To analyze the relevance of miR-27a-5p induction in a pathological context, we cocultured under transwell condition resting NK cells and the prototypic SH-SY5Y NB cell line, which we described to induce a TGF-β1-mediated downregulation of CX_3CR1 surface expression (12). Accordingly with our previous published data (12), NB conditioning resulted in a significant downregulation of CX_3CR1 expression at both protein and mRNA level. Importantly, this modulatory effect matched with a significant increase of miR-27a-5p as compared to unconditioned NK cells (**Figure 6**).

DISCUSSION

A previous study by our group reported that TGF-β1 released by NB cells is able to modify the chemokine receptor repertoire of NK cells (12). In particular, it downregulates CX_3CR1 expression in resting NK cells. In the present article, we show that TGF-β1-induced miR-27a-5p directly modulates the expression of CX_3CR1 mRNA at the post-transcriptional level. Increment of miR-27a-5p expression is due to upregulation of the miR-23a-27a-24-2 cluster, which contains, among others, miR-27a-5p. Importantly, we observed that this regulatory mechanism also occurs in NK cells during NB-conditioning.

All our results were obtained using unfractioned blood NK cells from healthy donors, mostly represented (more than 90%) by the $CD56^{dim}$ population (7, 26). Since $CD56^{bright}$ NK cells

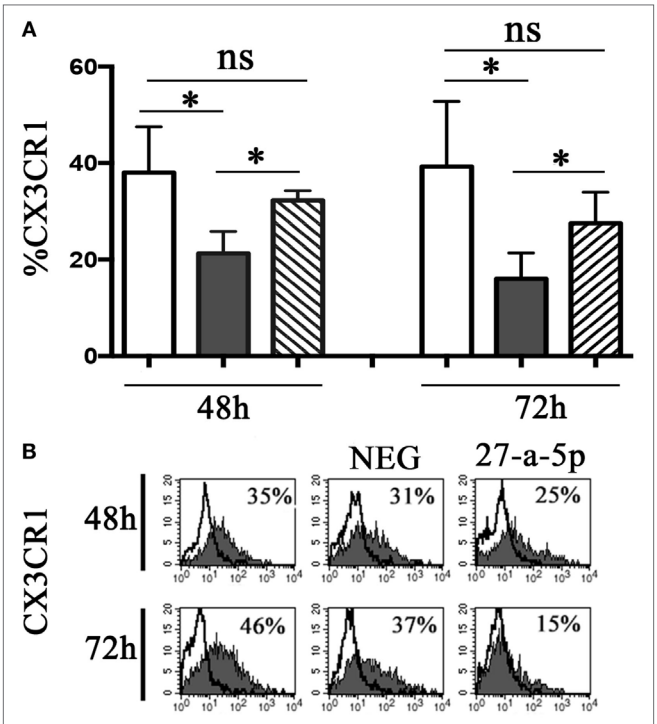

FIGURE 5 | miR-27a-5p-induced downregulation of CX_3CR1 surface expression. **(A)** CX_3CR1^+ HEK293T cells (clone #124) untrasfected (white bar), transfected with miR-27a-5p mimic (black bar), or miRNA negative control (stripped bar) were analyzed by flow cytometry for the expression of CX_3CR1 at the indicated time intervals. Average of four independent experiments. 95% confidence intervals and significance ($^*p < 0.05$) are shown. ns, non-significant. **(B)** Representative cytofluorimetric analysis of CX_3CR1 surface expression. Values inside each histogram indicate the percentage (%) of positive cells. NEG: miRNA negative control.

virtually do not express CX_3CR1, further experiments should clarify whether miR-27a-5p might contribute to their costitutive $CX_3CR1^{low/neg}$ phenotype.

The downregulation of CX_3CR1 has important consequences on NK cells function, as CX_3CR1, with other chemokine receptors, drives, at steady state, NK cell localization in peripheral tissues as well as their migration under inflammatory conditions (27). In particular, it has been shown that the relative expression of CX_3CR1 and CXCR4 regulates NK cell migration within the BM and their egress from it (28, 29). Moreover, in a model of experimental autoimmune encephalomyelitis (EAE), CX_3CR1/fractalkine axis specifically recruits NK cells into the brain (30). Indeed in CX_3CR1-deficient mice with EAE the amount of NK cells in the inflamed CNS cells was markedly reduced, as compared to wild-type mice, whereas that of other cell types, including T, NKT cells, and monocyte/macrophages, did not show significant variation (30). Importantly, recruitment of CX_3CR1+ NK cells toward the inflamed CNS ameliorated the EAE disease severity, since mature NK cells showed a higher cytolytic activity against autoreactive CD4+ T cells (21).

Interestingly, CX_3CR1/CX_3CL1 axis, other than regulate NK cell migration, increases NK cell responsiveness to CCL4 (MIP1β) and CXCL8 (IL-8) (31) and promotes IFN-γ production and cytotoxicity by NK cells (32).

The functional relevance of CX_3CR1 in NK cells tropism and activity is further underlined by the identification of viruses producing viral chemokines able to bind CX_3CR1: vMIP-II, encoded by the Kaposi's sarcoma-associated herpesvirus, which inhibits naive migration of $CD56^{dim}$ NK cells blocking the binding of CX_3CL1 (33), and vCXCL1, encoded by human cytomegalovirus, which induces NK and neutrophils migration, presumably to facilitate a neutrophil-mediated viral dissemination (34).

CX_3CR1 expression also has a pivotal role in tumors, conditioning migration and adhesion of tumor cells, and tumor invasiveness and metastasis (35). In particular, CX_3CR1 expression in pancreatic and prostate cancer cells increases invasiveness and metastasis to neuronal tissues (36). Interestingly, however, while CX_3CR1 expression in cancer is generally associated with invasiveness and metastasis, the coexpression of CX_3CR1 and CX_3CL1 by the same cell type, as occurs in human colorectal cancer cells, acts as a retention factor, limiting tumor spreading to metastatic sites (37).

Due to the wide properties of CX_3CR1, we can argue that the receptor downregulation caused by TGF-β1 in mature cytolytic NK cells might severely hamper their recruitment and functions at tumor sites. This might occur thanks the TGF-β1-induced upregulation of the miR-23a-27a-24-2 cluster in NK cells, which in turn causes miR-27a-5p upregulation and the consequent CX_3CR1 downregulation. It is likely that a tight control of the miR-23a-27a-24-2 cluster expression is necessary, given that its deregulation has been reported in several tumors and other diseases (38). Interestingly, TGF-β1 has been shown to be responsible for upregulation of the miR-23a-27a-24-2 cluster in hepatocellular carcinoma cells (39), in lung adenocarcinoma cells (40) as well as in CD8 T cells (41). In these lymphocytes, additionally, upregulation of the cluster has been associated with inhibition of IFN-γ expression and reduced cytotoxicity (41). miR-27a-5p has been also reported to downregulate perforine 1 (Prf1) and granzyme B (GzmB) expression in resting and IL-15-activated NK cells (42), thus hampering NK cells cytotoxicity. NK92 and primary NK cells overexpressing miR-27a-5p showed a reduced cytotoxicity but unmodified levels of the activating receptors NKG2D, NKp30, NKp44, and NKp46. Moreover, knockdown of miR-27a-5p in NK cells increased *in vitro* cytotoxicity and decreased tumor growth in a human tumor xenograft model (42). Overall, these data suggest that miR-23a-27a-24-2 cluster could be an important target of TGF-β1, possibly acting as an intermediate inducer of its effects. miR-27a-5p, a product of this cluster, is able to regulate the expression of multiple targets crucial for NK cells function. Thus, it appears as a key node for NK activity control, being able to dampen NK cell recruitment, cytotoxicity, and IFN-γ production, potentially representing, in perspective, an interesting cancer therapeutic target.

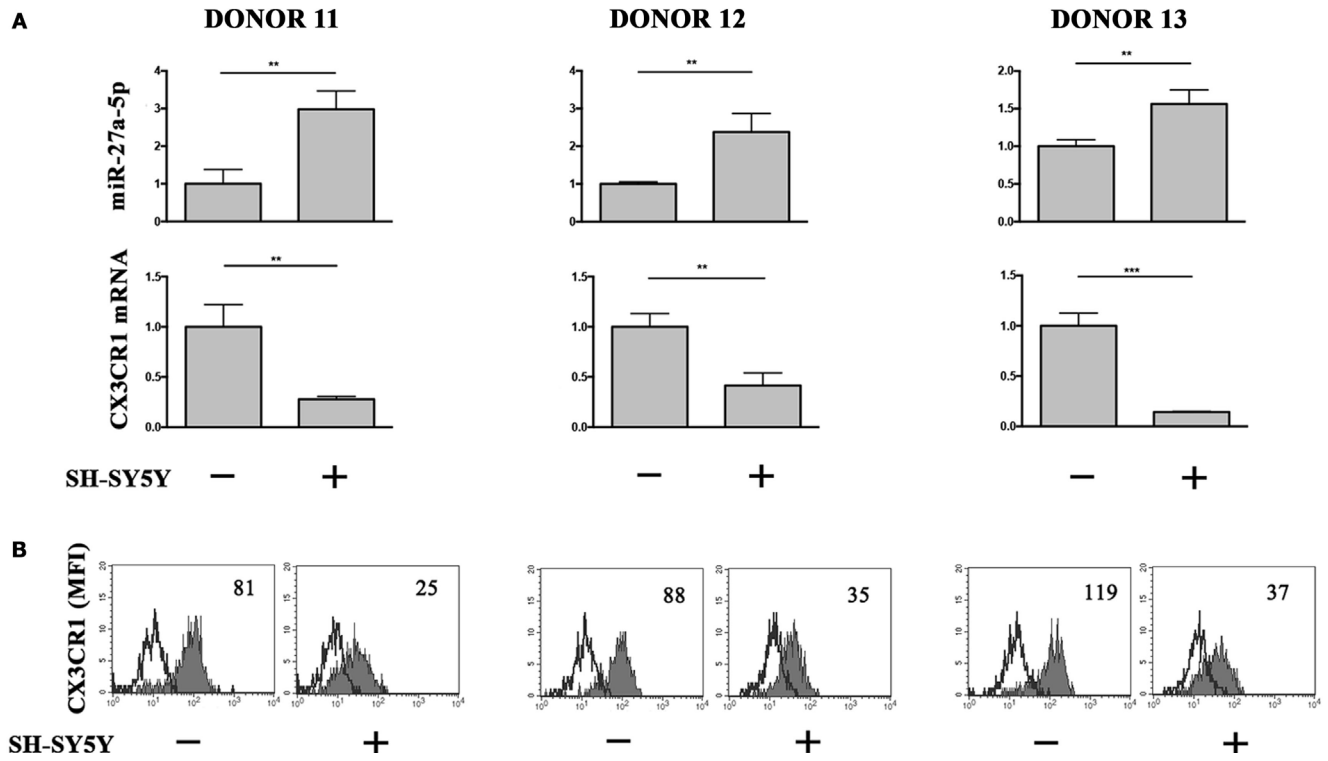

FIGURE 6 | miR-27a-5p and CX_3CR1 expression in NK cells cocultured under transwell conditions with SH-SY5Y neuroblastoma cell line. **(A)** miR-27a-5p and CX_3CR1 mRNA expression of SH-SY5Y-conditioned NK cells from three healthy donors (donors 11, 12, and 13). Data in triplicate from each donor are shown. RNU44 and GAPDH were used as reference controls, respectively. $^{**}p < 0.01$; $^{***}p < 0.001$. **(B)** Cytofluorimetric analysis of CX_3CR1 expression in NK cells from the same donors. White profiles refer to cells incubated with isotype-matched mAbs. Value inside each histogram indicates the median fluorescence intensity (MFI).

AUTHOR CONTRIBUTIONS

SR, CB, and RC designed the study. SR, FC, AD, BC, FR, FL, and RC performed the experiments, analyzed, and interpreted data. SR, FC, AM, CB, and RC wrote the manuscript. All the authors read and approved the final article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00868/full#supplementary-material.

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